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Inventors : Nozer M. Mehta et al.
Title : DIRECT EXPRESSION OF PEPTIDES INTO CULTURE MEDIA
Assignee : Unigene Laboratories, Inc.

Enclosed herewith please find the following documents in the above-identified application for United States Letters Patent:

76 Pages of Specification including Abstract and Claims
101 Numbered Claims Calculated as 101 Claims for Fee Purposes
17 Sheets of Drawing Containing Figures 1A to 17. (Informal)
XX Unexecuted Declaration and Power of Attorney
XX Priority is Claimed under 35 U.S.C. §119 of co-pending United States
Provisional Appln. S.N. 60/043,700, filed April 16, 1997.
Unexecuted Verified Statement Claiming Small Entity Status under 37
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The Patent and Trademark Office is authorized to charge the above and any underpayment, and any other fee due at any time during prosecution of this application that is not paid at the time due, to Deposit Account No. 15-0700.

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DIRECT EXPRESSION OF PEPTIDES INTO CULTURE MEDIARELATED APPLICATION

5 This application is a continuation-in-part of
co-pending provisional U.S. patent application serial
number 60/043,700 filed April 16, 1997.

FIELD OF THE INVENTION

10 The present invention relates to direct
expression of a peptide product into the culture medium
of genetically engineered host cells expressing the
peptide product. More particularly, the invention
relates to expression vectors, host cells and/or
15 fermentation methods for producing a peptide product that
is excreted outside the host into the culture medium in
high yield. In some embodiments, the invention relates
to direct expression of a peptide product having C-
terminal glycine which is thereafter converted to an
amidated peptide having an amino group in place of said
glycine.

DESCRIPTION OF THE RELATED ART

20 Various techniques exist for recombinant
production of peptide products, i.e. any compound whose
molecular structure includes a plurality of amino acids
linked by a peptide bond. A problem when the foreign
peptide product is small is that it is often readily
25 degradable by endogenous proteases in the cytoplasm or
periplasm of the host cell that was used to express the
peptide. Other problems include achieving sufficient
yield, and recovering the peptide in relatively pure form

without altering its tertiary structure (which can undesirably diminish its ability to perform its basic function). To overcome the problem of small size, the prior art has frequently expressed the peptide product of interest as a fusion protein with another (usually larger) peptide and accumulated this fusion protein in the cytoplasm. The other peptide may serve several functions, for example to protect the peptide of interest from exposure to proteases present in the cytoplasm of the host. One such expression system is described in Ray et al., *Bio/Technology*, Vol. 11, pages 64-70, (1993).

However, the isolation of the peptide product using such technology requires cleavage of the fusion protein and purification from all the peptides normally present in the cytoplasm of the host. This may necessitate a number of other steps that can diminish the overall efficiency of the process. For example, where a prior art fusion protein is accumulated in the cytoplasm, the cells must usually be harvested and lysed, and the cell debris removed in a clarification step. All of this is avoided in accordance with the present invention wherein the peptide product of interest is expressed directly into, and recovered from, the culture media.

In the prior art it is often necessary to use an affinity chromatography step to purify the fusion protein, which must still undergo cleavage to separate the peptide of interest from its fusion partner. For example, in the above-identified *Bio/Technology* article, salmon calcitonin precursor was cleaved from its fusion partner using cyanogen bromide. That cleavage step necessitated still additional steps to protect cysteine sulfhydryl groups at positions 1 and 7 of the salmon calcitonin precursor. Sulfonation was used to provide

protecting groups for the cysteines. That in turn altered the tertiary structure of salmon calcitonin precursor requiring subsequent renaturation of the precursor (and of course removal of the protecting groups).

The peptide product of the invention is expressed only with a signal sequence and is not expressed with a large fusion partner. The present invention results in "direct expression". It is expressed initially with a signal region joined to its N-terminal side. However, that signal region is post-translationally cleaved during the *secretion* of the peptide product into the periplasm of the cell. Thereafter, the peptide product diffuses or is otherwise excreted from the periplasm to the culture medium outside the cell, where it may be recovered in proper tertiary form. It is not linked to any fusion partner whose removal might first require cell lysing denaturation or modification, although in some embodiments of the invention, sulfonation is used to protect cysteine sulfhydryl groups during purification of the peptide product.

Another problem with the prior art's accumulation of the peptide product inside the cell, is that the accumulating product can be toxic to the cell and may therefore limit the amount of fusion protein that can be synthesized. Another problem with this approach is that the larger fusion partner usually constitutes the majority of the yield. For example, 90% of the production yield may be the larger fusion partner, thus resulting in only 10% of the yield pertaining to the peptide of interest. Yet another problem with this approach is that the fusion protein may form insoluble

inclusion bodies within the cell, and solubilization of the inclusion bodies followed by cleavage may not yield biologically active peptides.

5 The prior art attempted to express the peptide
together with a signal peptide attached to the N-terminus
to direct the desired peptide product to be secreted into
the periplasm (see EP 177,343, Genentech Inc.). Several
signal peptides have been identified (see Watson, M.
Nucleic Acids Research, Vol 12, No.13, pp: 5145-5164).
10 For example, Hsiung et al. (Biotechnology, Vol 4,
November 1986, pp: 991-995) used the signal peptide of
outer membrane protein A (OmpA) of *E. coli* to direct
certain peptides into the periplasm. Most often,
peptides secreted to the periplasm frequently tend to
15 stay there with minimal excretion to the medium. An
undesirable further step to disrupt or permeabilize the
outer membrane may be required to release sufficient
amounts of the periplasmic components. Some prior art
attempts to excrete peptides from the periplasm to the
20 culture media outside the cell have included compromising
the integrity of the outer membrane barrier by having the
host simultaneously express the desired peptide product
containing a signal peptide along with a lytic peptide
protein that causes the outer membrane to become
25 permeable or leaky (U.S. Patent No. 4,595,658). However,
one needs to be careful in the amount of lytic peptide
protein production so as to not compromise cellular
integrity and kill the cells. Purification of the
peptide of interest may also be made more difficult by
30 this technique.

 Aside from outer membrane destabilization
techniques described above there are less stringent means
of permeabilizing the outer membrane of gram negative

bacteria. These methods do not necessarily cause destruction of the outer membrane that can lead to lower cell viability. These methods include but are not limited to the use of cationic agents (Martti Vaara.,
5 Microbiological Reviews, Vol. 56, pages 395-411 (1992)) and glycine (Kaderbhai et al., Biotech. Appl. Biochem, Vol. 25, pages 53-61 (1997)) Cationic agents permeabilize the outer membrane by interacting with and causing damage to the lipopolysaccharide backbone of the outer membrane.
10 The amount of damage and disruption can be non lethal or lethal depending on the concentration used. Glycine can replace alanine residues in the peptide component of peptidoglycan. Peptidoglycan is one of the structural components of the outer cell wall of gram negative
15 bacteria. Growing *E. coli* in high concentration of glycine increases the frequency of glycine-alanine replacement resulting in a defective cell wall, thus increasing permeability.

Another prior art method of causing excretion of a desired peptide product involves fusing the product to a carrier protein that is normally excreted into the medium (hemolysin) or an entire protein expressed on the outer membrane (e.g. ompF protein). For example, human β -endorphin can be excreted as a fusion protein by *E.*
20 *coli* cells when bound to a fragment of the ompF protein (EMBO J., Vol 4, No. 13A, pp:3589-3592, 1987). Isolation of the desired peptide product is difficult however, because it has to be separated from the carrier peptide, and involves some (though not all) of the drawbacks
25 associated with expression of fusion peptides in the cytoplasm.
30

Yet another prior art approach genetically alters a host cell to create new strains that have a

permeable outer membrane that is relatively incapable of retaining any periplasmic peptides or proteins. However, these new strains can be difficult to maintain and may require stringent conditions which adversely affect the yield of the desired peptide product.

Raymond Wong et al. (U.S. Patent No. 5,223,407) devised yet another approach for excretion of peptide products by making a recombinant DNA construct comprising DNA coding for the heterologous protein coupled in reading frame with DNA coding for an ompA signal peptide and control region comprising a tac promoter. This system reports yields significantly less than those achievable using the present invention.

Although the prior art may permit proteins to be exported from the periplasm to the media, this can result in unhealthy cells which cannot easily be grown to the desirable high densities, thus adversely affecting product yield.

The present invention seeks to produce peptide in high yield with an efficient expression vector, and to provide high yield culturing techniques and other improvements which permits high yield recovery of excreted peptide of interest from the culture media, without overly disrupting the integrity of the cell membrane.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to have a peptide product accumulate in good yield in the medium in which peptide-producing host cells are growing. This is advantageous because the medium is relatively free of many cellular peptide contaminants.

It is another object of the invention to provide an improved fermentation process for increasing the yield of a peptide product expressed by genetically engineered host cells.

It is another object of the invention to provide genetically engineered host cells that are particularly useful in expressing the novel expression vectors of the invention.

It is another object of the invention to provide a host cell which is particularly suited to the production of salmon calcitonin precursor, regardless of the expression vector utilized for expression of salmon calcitonin.

It is a further object of the invention to provide improved methods for the production of amidated peptides utilizing precursor peptides having C-terminal glycines, which precursors are amidated following direct expression into the culture medium in accordance with the invention.

In one embodiment, the invention provides an expression vector comprising: (a) a coding region with nucleic acids coding for a peptide product coupled in reading frame 3' of nucleic acids coding for a signal peptide; and (b) a control region linked operably with the coding region, said control region comprising a plurality of promoters and at least one ribosome binding site, wherein at least one of said promoters is tac.

Host cells transformed or transfected with the vector are provided, as are methods of direct expression of the peptide product by culturing such host cells.

5 In another embodiment, the invention provides a host cell transformed with an expression vector which comprises a gene for expressing salmon calcitonin precursor, or calcitonin gene related peptide precursor, said host cell being *E. coli* strain BLR; and methods of culturing the same to obtain said precursor in the media.

10 In another embodiment, the invention provides a method of producing an amidated peptide product by producing a precursor having a C-terminal glycine using any of the vectors, hosts, or fermentation processes reported herein; and thereafter converting said glycine to an amino group to produce a peptide amide.

15 In another embodiment, the invention provides a method for direct expression of a peptide product into a culture medium comprising the steps of: (a) culturing, in said medium, genetically engineered host cells which
20 express said peptide product together with a signal peptide under conditions wherein growth of said host cells is controlled to stay within a range of 0.05 to 0.20 doublings per hour; wherein an inducer is present during some of said period of controlled growth; and (b)
25 recovering said peptide product from the culture medium after intracellular cleavage of the signal peptide.

30 In another embodiment, glycine is added to the medium during the course of direct expression fermentation, in order to increase the permeability of the outer membrane and enhance excretion of the peptide product.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show a schematic diagram of the construction of the pSCT-016B vector (1A) which is used in the construction of the pSCT-018D vector (1B) which is in turn used in the construction of vector pSCT-025.

Figure 2 shows a schematic diagram of the construction of the pSCT-019 vector. The LAC-OMPASCTGLY cassette used in the construction of pSCT-025 was made by polymerase chain reaction (PCR) amplification of a portion of pSCT-019.

Figure 3 shows a schematic diagram of the construction of the pSCT-025 vector which was used in the construction of vectors pSCT-029A, pSCT-025A, pSCT-037 and pSCT-038.

Figure 4 shows a schematic diagram of the construction of the pSCT-029A vector which was used in the construction of vector pSCT-038 and pSCT-034. In addition, pSCT-029A was used to transform *E. coli* BLR and produce the novel digenic UGL 165 clone.

Figure 5 shows a schematic diagram of the construction of the pSEC-E vector which was used in the construction of vector pSEC-EY.

Figure 6 shows a schematic diagram of the construction of the pPRLA4 vector which was used in the construction of vector pSEC-EY.

Figure 7 shows a schematic diagram of the construction of the pSEC-EY vector which was used in the construction of vector pSCT-037 and pSCT-038.

Figure 8 shows a schematic diagram of the construction of pSCT-037 and pSCT-038 vectors. pSCT-037 was used to transform *E. coli* BLR and produce the monogenic UGL 702 clone. pSCT-038 was used to transform

E. coli BLR and produce the digenic UGL 703 clone. pSCT-038 contains two copies of the OmpA-sCTgly operon (encoding the OmpA signal together with salmon calcitonin precursor) making it a novel digenic expression vector.

5 It also contains copies of genes encoding two sec machinery proteins which enhance translocation across the inner membrane into the periplasmic space. The following is a list of abbreviations used in describing this vector of Figure 8:

10 TAC - Hybrid promoter of tryptophan E and lac operator sequences;
LAC P/O - Region containing lac promoter and lac operator of β galactosidase gene;
LAC-IQ - Gene coding for the lac repressor that
15 binds to operator region of lac promoter and tac promoter. IPTG competes with lac repressor and inhibits binding of lac repressor to operator region of both tac promoter and lac promoter, thus inducing said promoters.
20 TRP P/O - Promoter operator region of tryptophan E gene;
OMPA-SCTGLY - Gene fusion containing secretory signal sequence of the outer membrane protein A gene and the coding sequence for glycine
25 extended salmon calcitonin (the salmon calcitonin precursor);
SEC-E (also known as "PrlG") - Gene coding for secretion factor E of *E. coli*. It combines with prlA [also known as secY] or prlA-4 to
30 form the inner membrane translocation domain of the sec pathway by which signal sequence containing proteins are translocated from the cytoplasm to the periplasm;

PRLA-4 - Mutant allele of *prlA* gene;

RRNB T1-T2 - Tandem transcription terminators 1 and 2 from *E. coli* Ribosomal protein gene; and

KAN-R - Kanamycin resistance gene.

5 Figure 9 shows a schematic diagram of the pSCT-034 vector which was used to transform *E. coli* BLR and produce the trigenic UGL 168 clone.

10 Figure 10 shows cell growth and sCTgly production of UGL 165 clone (plasmid pSCT029A in *E. coli* BLR) over time after a typical 1 liter fermentation in the presence of inducer. Cell growth was measured by light absorbance at a wavelength of 600 nm. sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Time zero indicates the time
15 at which inducer is first added to the culture medium where host cells of the invention are being cultured. Figure 10 shows that most of the sCTgly production by UGL 165 occurs between 20 and 25.5 hours after inducer is first added to the culture media where host cells of the
20 invention are being cultured.

 Figure 11 shows cell growth and sCTgly production of UGL 703 clone (plasmid pSCT 038 in *E. coli* BLR) over time after a typical 1 liter fermentation in the presence of inducer. Cell growth was measured by
25 light absorbance at a wavelength of 600 nm. sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Time zero indicates the time at which inducer is first added to the culture medium where host cells of the invention are being cultured.
30 Figure 11 shows that most of the sCTgly production by UGL 703 occurs between 20 and 26 hours after culture in the presence of inducer.

Figure 12 shows a comparison of the cell growth of UGL 172 clone (plasmid pSCT 025 in *E. coli* BLR), UGL 165 clone and UGL 168 clone (plasmid pSCT 034 in *E. coli* BLR) in a typical 1 liter fermentation over time after incubation in the presence of inducer as measured by absorbance at a wavelength of 600 nm. Figure 12 shows no significant differences in cell growth rates of UGL 165 and UGL 172 while UGL 168 shows a slight reduction in cell growth rate in this particular experiment.

Figure 13 shows a comparison of sCTgly production by UGL 172 clone, UGL 165 clone and UGL 168 clone over time in a typical 1 liter fermentation after incubation in the presence of inducer reported as mg of sCTgly excreted per liter of incubation medium. Figure 13 shows that the digenic UGL 165 clone is best suited for production of sCTgly with the trigenic clone being second best over the monogenic UGL 173 clone.

Figures 14A and 14B show a comparison of sCTgly production (14A) and cell growth (14B) over time after a typical 1 liter fermentation in the presence of inducer by UGL 165 clone and either in the presence or absence of oxygen supplementation to the air feed. Cell growth was measured as g of wet cell weight per liter of incubation media. sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Figures 14A and 14B show that added oxygen in the fermentation medium is not critical to cell growth of UGL 165 but is very important in increasing the production of sCTgly.

Figures 15A and 15B show a comparison of the cell growth (15A) and sCTgly production (15B) over time after a typical 1 liter fermentation in the presence of inducer by the *E. coli* strains WA837 and BLR where each strain is expressing the pSCT-029A vector (UGL164 and

UGL165 respectively). Cell growth was measured by light absorbance at a wavelength of 600 nm. sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Figures 15A and 15B show that the BLR *E. coli* strain is more suited for sCTgly production than the WA837 *E. coli* strain.

Figure 16 shows a comparison of sCTgly production after a typical 1 liter fermentation in the presence of inducer by the *E. coli* strains WA837, BLR, BL21 and B834, where each strain is expressing the pSCT-029A vector (UGL164, UGL165, UGL167 and UGL166 respectively). sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Figure 16 shows that the *E. coli* BLR strain is more suited for sCTgly production than each of the WA837, BL21 and B834 *E. coli* strains.

Figure 17 shows a comparison of the best sCTgly production observed from different experiments after 1 liter fermentations in the presence of inducer by UGL 165 (pSCT-029A in BLR), UGL 168 (pSCT-034 in BLR), UGL 172 (pSCT-025 in BLR), UGL 702 (pSCT-037 in BLR) and UGL 703 (pSCT-038 in BLR) clones. sCTgly production was reported as mg of sCTgly excreted per liter of incubation medium. Figure 17 shows that the digenic UGL 703 and UGL 165 clones are more suited for sCTgly production than the monogenic UGL 172 and UGL 702 clones and the trigenic UGL 168 clone. Figure 17 also shows that the digenic UGL 703 clone that expresses secretion factors is more suited for sCTgly production than the digenic UGL 165 clone which does not express secretion factors.

DETAILED DESCRIPTION OF THE INVENTION

The present invention permits peptide product yields in excess of 100 mg per liter of media. It does so with novel expression vectors, novel hosts (as
5 transformed, transfected or used in accordance with the invention), novel fermentation processes, or a combination of two or more of the foregoing.

Overview of a Preferred Expression Vector

In one embodiment, the present invention
10 provides an expression vector which comprises a coding region and a control region. The coding region comprises nucleic acids for a peptide product of interest coupled in reading frame downstream from nucleic acids coding for a signal peptide. The control region is linked operably
15 to the coding region and comprises a plurality of promoters and at least one ribosome binding site, wherein at least one of the promoters is selected from the group consisting of tac and lac.

Preferably, the vector comprises a plurality of
20 transcription cassettes placed in tandem, each cassette having the control region and the coding region of the present invention. Such a digenic vector or multigenic vector is believed to provide better expression than would a dicistronic or multicistronic expression vector.
25 This is a surprising improvement over dicistronic or multicistronic expression which is not believed to be suggested by the prior art.

The vector can optionally further comprise nucleic acids coding for a repressor peptide which
30 represses operators associated with one or more of the promoters in the control region, a transcription terminator region, a selectable marker region and/or a

region encoding at least one secretion enhancing peptide. Alternatively, in some embodiments, nucleic acids coding for a repressor peptide and a secretion enhancing peptide may be present on a separate vector co-expressed in the same host cell as the vector expressing the peptide product.

Specific examples of constructed expression vectors, and methods for constructing such expression vectors are set forth infra. Many commercially available vectors may be utilized as starting vectors for the preferred vectors of the invention. Some of the preferred regions of the vectors of the invention may already be included in the starting vector such that the number of modifications required to obtain the vector of the invention is relatively modest. Preferred starting vectors include but are not limited to pSP72 and pKK233-2.

It is believed that the novel vectors of the invention impart advantages which are inherent to the vectors, and that those unexpected advantages will be present even if the vectors are utilized in host cells other than the particular hosts identified as particularly useful herein, and regardless of whether the improved fermentation process described herein is utilized.

Likewise, in certain embodiments, particular host cells are identified as being particularly useful in the expression of peptides such as salmon calcitonin precursor and calcitonin gene related peptide precursor. The advantages imparted by specifically utilizing the particular host cells identified herein are believed to exist regardless of whether the expression vector is one of the novel vectors described herein or whether the

novel fermentation process described herein is utilized. In other words, it is believed that these host cells will impart significant unexpected advantages even utilizing prior art fermentation or prior art vectors.

5 The novel fermentation process is believed to provide increased yield because of inherent advantages imparted by the fermentation process. It is believed that these advantages will be present regardless of whether the preferred host cells and/or novel vectors
10 described herein are utilized.

Notwithstanding the foregoing, one preferred embodiment of the invention simultaneously utilizes the improved expression vectors of the invention transformed into the particularly identified host cells of the
15 invention and expressed utilizing the preferred fermentation invention described herein. When all three of these inventions are used in combination, it is believed that a significant enhancement of yield and recovery of product can be achieved relative to the prior
20 art.

The control region

The control region is operably linked to the coding region and comprises a plurality of promoters and at least one ribosome binding site, wherein at least one
25 of the promoters is selected from the group consisting of lac and tac. It has surprisingly been found that the foregoing combination of promoters in a single control region significantly increases yield of the peptide product produced by the coding region (as described in
30 more detail infra). It had been expected that two such promoters would largely provide redundant function, and not provide any additive or synergistic effect.

Experiments conducted by applicants have surprisingly shown a synergy in using the claimed combination of promoters. Other promoters are known in the art, and may be used in combination with a tac or lac promoter in accordance with the invention. Such promoters include but are not limited to lpp, ara B, trpE, gal K.

Preferably, the control region comprises exactly two promoters. When one of the promoters is tac, it is preferred that the tac promoter be 5' of another promoter in the control region. When one of the promoters is lac, the lac promoter is preferably 3' of another promoter in the control region. In one embodiment, the control region comprises both a tac promoter and a lac promoter, preferably with the lac promoter being 3' of the tac promoter.

The coding region

The coding region comprises nucleic acids coding for a peptide product of interest coupled in reading frame downstream from nucleic acids coding for a signal peptide whereby the coding region encodes a peptide comprising, respectively, from N terminus to C terminus the signal and the peptide product. Without intending to be bound by theory, it is believed that the signal may provide some protection to the peptide product from proteolytic degradation in addition to participating in its secretion to the periplasm.

Many peptide signal sequences are known and may be used in accordance with the invention. These include signal sequences of outer membrane proteins of well-characterized host cells, and any sequences capable of translocating the peptide product to the periplasm and of being post-translationally cleaved by the host as a

result of the translocation. Useful signal peptides include but are not limited to Omp A, pel B, Omp C, Omp F, Omp T, β -la, Pho A, Pho S and Staph A.

5 The peptide product is preferably small enough so that, absent the present invention, it would usually require a fusion partner using prior art technology. Typically, the peptide product has a molecular weight of less than 10 KDa. More preferably, the peptide product has a C-terminal glycine, and is used as a precursor to
10 an enzymatic amidation reaction converting the C-terminal glycine to an amino group, thus resulting in an amidated peptide. Such a conversion is described in more detail infra. Numerous biologically important peptide hormones and neurotransmitters are amidated peptides of this type.
15 For example, the peptide product coded by the coding region may be salmon calcitonin precursor or calcitonin gene related peptide precursor, both of which have C-terminal glycines and both of which may be enzymatically amidated to mature salmon calcitonin or mature calcitonin
20 gene related peptide. Other amidated peptides that may be produced in accordance with the invention include but are not limited to growth hormone releasing factor, vasoactive intestinal peptide and galanin. Other amidated peptides are well known in the art.
25 Analogous of parathyroid hormone could also be produced in accordance with the invention. For example, a peptide having the first 34 amino acids of parathyroid hormone can provide a function similar to that of parathyroid hormone itself, as may an amidated version of
30 the 34 amino acid analog. The latter may be produced by expressing, in accordance with one or more of the expression systems and methods described herein, the first 34 amino acids of parathyroid hormone, followed by

glycine-35. Enzymatic amidation as disclosed herein could then convert the glycine to an amino group.

While preferred embodiments of the direct expression system described herein produce peptides having C-terminal glycine, it is believed that any peptide will enjoy good yield and easy recovery utilizing the vectors, hosts and/or fermentation techniques described herein.

Other Optional Aspects of a Preferred Vector of The Invention or of Other Vectors to be Expressed in the Same Host as the Vector of the Invention

Repressor

Optionally, the preferred vector of the present invention may contain nucleic acids coding for a repressor peptide capable of repressing expression controlled by at least one of the promoters.

Alternatively, however, the nucleic acids coding for a repressor peptide may be present on a separate vector in a host cell with the vector of the present invention.

Appropriate repressors are known in the art for a large number of operators. Preferably, the nucleic acids coding for the repressor encode a lac repressor in preferred embodiments of the invention because it represses the lac operator that is included with both tac and lac promoters, at least one of which promoters is always present in preferred vectors of the invention.

Selectable marker

It is preferred that any of a large number of selectable marker genes (e.g. a gene encoding kanamycin resistance) be present in the vector of the present

invention. This will permit appropriate specific selection of host cells that are effectively transformed or transfected with the novel vector of the invention.

Secretion enhancing peptide

5 Nucleic acids coding for at least one secretion enhancing peptide are optionally present in the vector of the present invention. Alternatively, the nucleic acids coding for a secretion enhancing peptide may be present on a separate vector expressed in the same host cell as
10 the vector encoding the peptide product. Preferably, the secretion enhancing peptide is selected from the group consisting of SecY (prlA) or prlA-4. It is pointed out that SecY and prlA are identical, the two terms being used as synonyms in the art. prlA-4 is a known
15 modification of prlA and has a similar function. Another preferred secretion enhancing peptide is SecE also known as "prlG", a term used as a synonym for "SecE". Most preferably, a plurality of secretion enhancing peptides are encoded, at least one of which is SecE and the other
20 of which is selected from the group consisting of SecY (prlA) and prlA-4. The two are believed to interact to aid translocation of the peptide product from cytoplasm to periplasm. Without intending to be bound by theory, these secretion enhancing peptides may help protect the
25 peptide product from cytoplasmic proteases in addition to their secretion enhancing functions.

Host cell

The present invention also provides a host cell transformed or transfected with any of the vectors of the
30 present invention. Preferably, the host cell is a bacterial cell. More preferably, the host cell is a gram

negative bacterial cell. Still more preferably, the host cell is *E. coli*. More preferably, the *E. coli* is strain BLR, BL21 or WA837. Most preferably, the *E. coli* is strain BLR. Also most preferably, the host cell further expresses at least one secretion-enhancing peptide.

The present invention further provides a host cell transformed with an expression vector which comprises a gene for expressing salmon calcitonin precursor or Calcitonin Gene Related Peptide precursor, said host cell being *E. coli* strain BLR. It is believed that BLR expression of these two peptides will be particularly effective even where prior art vectors are used for the expression. In other words, it is not believed that the novel expression vectors reported herein are required for good expression of these two peptides in a BLR host.

Method of producing a heterologous peptide

Novel fermentation conditions are provided for growing host cells to very high cell densities under culture conditions which permit the diffusion or excretion of the peptide product into the culture medium in high yield.

Host cells useful in the novel fermentation include but are not limited to the host cells discussed supra, and/or host cells transformed or transfected with one or more of the novel expression vectors discussed supra. Other host cells genetically engineered to express peptide product together with a signal region may be used. The cells are placed in a fermenter which preferably includes appropriate means of feeding air or other gases, carbon source, and other components to the media and means for induction of the promoter.

Appropriate means for monitoring oxygen content, cell density, pH and the like are also preferred.

Applicants have found that significantly improved yield of peptide product directly expressed into the culture medium is obtained by carefully controlling the average cell growth rate within a critical range between 0.05 and 0.20 doublings per hour. It is preferred that this controlled growth begin in early lag phase of the culture. It is more preferable to maintain average cell growth rate during the fermentation period (i.e. the period during which growth is being controlled as set forth herein), between 0.10 and 0.15 doublings per hour, most preferably 0.13 doublings per hour. Growth rate may be controlled by adjusting any of the parameters set forth infra in the section entitled "Production of sCTgly (Fermentation)", specifically the formula equating the feed rate "Q" to numerous other parameters. Applicants have found that varying the rate of carbon source being fed to the fermenting cells is an advantageous method of maintaining the growth rate within the critical range. In order to maintain the growth rate relatively constant, the amount of carbon source feeding into the fermenter tends to increase proportionally to the growth in number of cells.

Applicants have also discovered that significantly improved yield can be obtained by providing inducer during said fermentation period of controlled growth. Like carbon source, feeding proper amounts of inducer involves increasing the rate of feed proportional to growth in number of cells. Since both carbon source and inducer feed preferably increase in a manner which is linked to cell growth, applicants have found that it is advantageous to mix feed and inducer together and to feed

the mixture of the two at the appropriate rate for
controlling cell growth (with the carbon source), thus
simultaneously maintaining a continuous feed of inducer
which stays at a constant ratio relative to the amount of
5 carbon source. However, it is of course possible to feed
carbon source and inducer separately. Even then,
however, if a chemical inducer that may be toxic to the
cells in large amounts is used, it is desirable that the
inducer and carbon source be added during each hour of
10 culturing in amounts such that the weight ratio of the
inducer added in any given hour to the carbon source
added in that same hour does not vary by more than 50%
from the ratio of the amount of inducer added during the
entirety of the fermentation process (controlled growth
15 period) to amount of carbon source added during the
entirety of the fermentation process. The 50% variance
is measured from the lower ratio of two ratios being
compared. For example, where the ratio of carbon source
to inducer for the entire fermentation is 2 to 1, the
20 ratio in any given hour is preferably no higher than 3 to
1 and no lower than 1.333 to 1. It is also possible to
induce one or more of the promoters during growth by
other means such as a shift in temperature of the culture
or changing the concentration of a particular compound or
25 nutrient.

When external carbon source feed is used as the
method of controlling cell growth, it is useful to wait
until any carbon sources initially in the media (prior to
external carbon feed) have been depleted to the point
30 where cell growth can no longer be supported without
initiating external carbon feed. This assures that the
external feed has more direct control over cell growth
without significant interference from initial (non-feed)

carbon sources. An oxygen source is preferably fed continuously into the fermentation media with dissolved oxygen levels being measured. An upward spike in the oxygen level indicates a significant drop in cell growth which can in turn indicate depletion of the initial carbon source and signify that it is time to start the external feed.

It has been unexpectedly found that peptide product yield increases as oxygen saturation of the fermentation media increases. This is true even though lower oxygen saturation levels are sufficient to maintain cell growth. Thus, during the entire fermentation process, it is preferred that an oxygen or oxygen enriched source be fed to the fermentation media, and that at least 20% and preferably at least 50% oxygen saturation be achieved. As used herein, "oxygen saturation" means the percentage of oxygen in the fermentation medium when the medium is completely saturated with ordinary air. In other words, fermentation media saturated with air has an "oxygen saturation" of 100%. While it is difficult to maintain oxygen saturation of the fermentation medium significantly above 100%, i.e. above the oxygen content of air, this is possible, and even desirable in view of higher oxygen content providing higher yields. This may be achieved by sparging the media with gases having higher oxygen content than air.

Significant yield improvement may be achieved by maintaining oxygen saturation in the fermentation medium at no lower than 70%, especially no lower than 80%. Those levels are relatively easy to maintain.

Faster agitation can help increase oxygen saturation. Once the fermentation medium begins to

thicken, it becomes more difficult to maintain oxygen saturation, and it is recommended to feed gases with higher oxygen content than air at least at this stage. Applicants have found that ordinary air can be sufficient to maintain good oxygen saturation until relatively late in the fermentation period. Applicants have supplemented the air feed with a 50% oxygen feed or a 100% oxygen feed later in the fermentation period. Preferably, the host cell is cultured for a period between 20 and 32 hours (after beginning controlled growth), more preferably between 22 and 29 hours, most preferably for about 24-27 hours.

Preferably, the host cells are incubated at a temperature between 20 and 35°C, more preferably between 28 and 32°C, more preferably between 29.5 and 30.5°C. A temperature of 30°C has been found optimal in several fermentations conducted by applicants.

Preferably, the pH of the culturing medium is between 6.0 and 7.5, more preferably between 6.6 and 7.0, with 6.78 - 6.83 (e.g. 6.8) being especially preferred.

In preferred embodiments, fermentation is carried out using hosts transformed with an expression vector having a control region that includes both a tac and a lac promoter and a coding region including nucleotides coding for a signal peptide upstream of nucleotides coding for salmon calcitonin precursor. Such an expression vector preferably includes a plurality, especially two, transcription cassettes in tandem. As used herein, the term "transcription cassettes in tandem" means that a control and coding region are followed by at least one additional control region and at least one additional coding region encoding the same peptide product as the first coding region. This is to be

distinguished from the dicistronic expression in which a single control region controls expression of two copies of the coding region. The definition will permit changes in the coding region that do not relate to the peptide product, for example, insertion, in the second transcription cassette, of nucleotides coding a different signal peptide than is coded in the first transcription cassette.

Numerous carbon sources are known in the art. Glycerol has been found effective. Preferred methods of induction include the addition of chemical inducers such as IPTG and/or lactose. Other methods such as temperature shift or alterations in levels of nutrient may be used. Other induction techniques appropriate to the operator or the promoter in the control region (or one of the plurality of promoters being used where more than one appears in the control region) may also be used.

It is typical that production of peptide product drops significantly at about the same time that growth of the cells in the fermentation media becomes unsustainable within the preferred growth rate discussed supra. At that point, fermentation is stopped, carbon source and inducer feed and oxygen flow are discontinued. Preferably, the culture is quickly cooled to suppress activity of proteases and thus reduce degradation of the peptide product. It is also desirable to modify pH to a level which substantially reduces proteolytic activity. When salmon calcitonin precursor is produced using preferred vectors and host cells of the invention, proteolytic activity decreases as pH is lowered. This acidification preferably proceeds simultaneously with

cooling of the media.¹ The preferred pH ranges are discussed in more detail infra. The same assay as is being used for measuring fermentation product can be used to measure degradation at different pH levels, thus
5 establishing the pH optimum for a given peptide and its impurities.

Recovery of the heterologous peptide

The present invention further provides a method for recovering the peptide product which comprises
10 separating the host cells from the culture medium and thereafter subjecting the culturing medium to at least one type of chromatography selected from the group consisting of gel filtration, ion-exchange (preferably cation exchange when the peptide is calcitonin), reverse-
15 phase, affinity and hydrophobic interaction chromatography. In a peptide containing cysteine residues, S-sulfonation may be carried out prior to or during the purification steps in order to prevent aggregation of the peptide and thereby increase the yield
20 of monomeric peptide. Preferably, three chromatography steps are used in the following order: ion exchange chromatography, reverse-phase chromatography and another ion exchange chromatography.

After fermentation is completed, the pH of the
25 culture medium is optionally altered to reduce the proteolytic activity. The assay used to measure product production can also be used to measure product degradation and to determine the best pH for stability. Where salmon calcitonin precursor is produced in
30 accordance with the invention, a pH between 2.5 and 4.0

¹or may be carried out after removal of the bacterial cells

is preferred, especially between 3.0 and 3.5. These pH ranges also are believed to aid retention of salmon calcitonin precursor on cation exchange columns, thus providing better purification during a preferred purification technique described herein.

Also optionally, the temperature of the medium, after fermentation is completed, is lowered to a temperature below 10°C, preferably between 3°C to 5°C, most preferably 4°C. This is also believed to reduce undesirable protease activity.

The present invention further provides a method of producing an amidated peptide product comprising the steps of: culturing, in a culture medium, any of the host cells of the present invention which express a peptide product having a C-terminal glycine; recovering said peptide product from said culture medium; amidating said peptide product by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine α -amidating monooxygenase, or peptidyl glycine α -hydroxylating monooxygenase. If peptidyl glycine α -amidating monooxygenase is not used hereinabove, and if the reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic. Amidated peptide may thereafter be recovered from the reaction mixture preferably utilizing the purification technique described infra in Example 6.

Preferably, the host cell is cultured in a culture medium in the presence of an inducer, while maintaining an average cell growth rate during culturing between 0.05 and 0.20 doublings per hour.

Experimental Details

pSCT-037 and pSCT-038 Cloning Strategy

The construction of pSCT-038 and pSCT-037 is comprised of eight parts converging to create the intermediate vectors needed to construct the final desired expression plasmids. All genes and fragments that were used or constructed outside of the text description for this project are listed in Table 1.

Part I Construction of pSCT-018D

The TAC promoter cartridge (Table 1) was subcloned into pGEM11ZF+ (Table 1) as a Hind III-Bam HI fragment creating pGEM11ZF+TAC. The pelB-sCTgly cas2 gene (Table 1) was ligated downstream of the tac promoter (in pGEM11ZF+TAC) into the Bam HI site to create the expression vector pSCT-013B. The tac-pelBsCTgly operon was cut from pSCT-013B using Hind III and Eco RI. This fragment was then ligated, along with a Hind III-Pst I adapter, into pSP72 (Table 1) creating pSCT-015B. The kanamycin resistance gene was then ligated into the Pst I site of pSCT-015B, creating pSCT-016B. The 5' coding and control region of the β -lactamase gene (ampicillin resistance) was deleted by cutting the vector with Pvu II and Fsp I followed by religation creating pSCT-017B. The T1-T2 transcription terminator from pSP72 T1-T2 (Table 1) was then cut and ligated into PSCT-017B using Sal I and Bgl II sites creating pSCT-018B. The LAC-IQ gene (Table 1) containing Bam HI and Eco RI sites was then ligated into the Bgl II - Eco RI sites of PSCT-018B creating pSCT-018D (see Figures 1A and 1B).

Part II Construction of pSCT-019

The lac P/O (Table 1) with sites Pst I and Hind III and a Hind III-Eco RI fragment from pSCT-013B (containing tac-pelBsCTgly) were inserted, in a three way
5 ligation, into the Pst I and Eco RI sites of pSP72 creating pSCT-015A. As described in the construction of pSCT-017B, the Kan-R gene was inserted and the β -lactamase gene was removed from pSCT-015A to create pSCT-016A and pSCT-017A. The lpp-lac-ompAsCTgly (Table I) Pst
10 I - Acc I fragment (17 base pairs of the 5' coding sequence of sCTgly are also present) was cut from pSP72-OMPA and ligated into the compatible sites in pSCT-017A to create pSCT-019 (see Figure 2).

Part III Construction of pSCT-025

15 A Bam HI fragment of the PCR amplified operon lac-ompAsCTgly (Table 1) was ligated into the Bam HI site of pSCT-018D, replacing the pelBsCTgly gene creating pSCT-023(-). The (-) signifies that the insert was
20 inserted in the reverse orientation in relation to the tac promoter. The lac-ompAsCTgly operon was then cut from pSCT-023(-), using Bam HI and Sal I, then ligated into the compatible sites of pSCT-017B creating pSCT-017 DELTA. The larger Bgl I-Bgl II fragment containing the
25 kanamycin resistance gene, tac-lac promoters and ompAsCTgly was cut from pSCT-017 DELTA and ligated into the Bgl I-Bam HI sites of pSCT-018D creating pSCT-025 (see Figure 3).

Part IV Construction of pSCT-029A

30 A PCR product of RRNB T1-T2-02 (Table 1), containing Xho I and Eco RI sites, was ligated into the Sal I - Eco RI sites of pSCT-025 creating pSCT-025A. A

Hind III-Sac I fragment cut from pSCT-025, containing the tac-lac-ompAsCTgly RRNB T1-T2 terminators gene cartridge, was ligated into the compatible sites of pSP72 creating pCPM-01. The pCPM-01A vector was constructed using the same method described for pSCT-025A. The pSCT-025A and pCPM-01A plasmids differ from the pSCT-025 and pSCT-01 plasmids in the types of restriction sites present upstream and downstream of the RRNB T1-T2 transcription terminator. An Xho I-Eco RI fragment, containing the tac-lac-ompAsCTgly RRNB T1-T2 terminators gene cartridge was cut from pCPM-01A and ligated into the Sal I-Eco RI sites of pSCT-025A creating the digenic expression vector pSCT-029A (Figure 4). The methods used to create pSCT-029A can be repeated to create additional polygenic expression vectors, as shown below for the construction of pSCT034.

Part V Construction of pSEC-E

The vector pCPM-01 was digested with Pst I and Xba I to excise the lac P/O, which was then ligated into the compatible sites of pSP72. The PCR amplified secE gene (Table I) containing Xba I and Bam HI cloning sites was ligated into the compatible sites of the ompAsCTgly T1-T2 cistron cloning vector (Table 1) creating pCM-SECE. The secE and T1-T2 terminators were cut from pCM-SECE as an Xba I-Eco RI fragment and ligated along with an Xba I-Sal I fragment, containing the lac P/O (cut from pSP72-lac), into the Sal I-Eco RI sites of pSP72 creating pSEC-E (see Figure 5)

Part VI Construction of pPRLA-4 (prlA-4 is a mutant allele of the prlA or secY gene)

The T1-T2 terminator region of the ompAsCTgly T1-T2 cistron vector was excised using Bam HI and Sma I, which was then ligated into the Pvu II and Bam HI sites of pSP72 creating the PRLA4-INT intermediate cloning vector. The lac P/O was cut from pSP72-LAC with Xba I and Sac I and ligated along with the prlA-4 PCR fragment (Table I), containing Xba I and Bam HI restriction sites, into the Sac I - Bam HI sites of PRLA4-INT creating pPRLA-4 (see Figure 6).

Part VII Construction of pSEC-EY

A synthetic oligonucleotide fragment containing the trpE P/O sequence (Table I) was subcloned into the Xho I-Xba I sites of pCPM-01A creating pCPM-08. The prlA-4 and T1-T2 sequences were cut from pPRLA-4 with Xba I and Xho I and ligated with a Sal I-Eco RI fragment from pSEC-E, containing the lac-secE-T1-T2 operon, into the Xba I and Eco RI sites of pCPM-08 creating pSEC-EY (see Figure 7).

Part VIII Construction of pSCT-037 and pSCT-038

The secE and prlA-4 coding region were cut from pSEC-EY with Xho I and Bgl II. The resulting fragment was then ligated into the Sal I-Bgl II sites of pSCT-025A and pSCT-029A creating pSCT-037 and pSCT-038 respectively (see Figure 8)

Construction of pSCT-034

pSCT-034 is a trigenic expression plasmid containing three copies of the Tac-Lac-ompAsCTgly RRNB T1-T2 transcription cassette. This vector (see Figure 9)

was constructed by inserting the described cartridge from pCPM-01A into Sal I and Eco RI sites of pSCT-029A adding a third copy of the expression cartridge. The method of construction is identical to the method for the construction of pSCT-029A from pCPM-01A and pSCT-025A. The 3' Sal I and Eco RI sites are recreated, providing the sites necessary for adding more copies of the cartridge.

TABLE 1

10	Cloning fragment	Component type	Origin or template
	pGEM 11 ZF(+)	Plasmid	Promega
	PGEX 1N	Plasmid	Pharmacia Biosciences
	pSP 72	Plasmid	Promega
	Tac promoter	tac promoter DNA block	Pharmacia Biosciences
15	pelB-sCTgly-cas2*	PCR amplified gene	pelB-sCTgly gene assembled from synthetic oligonucleotides
	Kanamycin resistance gene	Gene block	Pharmacia Biosciences
	RRNB T1-T2	PCR fragment	Ribosomal protein gene T1 and T2 transcription terminators from pKK 233-2
	pSP72 T1-T2	Subclone	Subcloned from above PCR fragment into pSP72
20	Lac repressor (LAC-IQ)	PCR amplified gene	pGEX 1-N plasmid
	Lac promoter/operator (P/O)	PCR amplified fragment	pGEM11ZF+
25	lpp-lac-ompAsCTgly (partial)	PCR amplified product (3' PCR primer contains first 17 nucleotides of sCTgly)	pIN IIIA
	pSP 72-ompA	Subclone of above fragment	Subcloned from above PCR fragment into pSP72

	lac-ompAsCTgly	PCR amplified operon	pSCT-019
	RRNB T1-T2-02	PCR amplified product	pSCT-025
	OMPASCTGLY T1-T2 CISTRON	PCR amplified product of ompAsCTgly subcloned into pSP72 T1-T2	pSCT-025 (template)
5	SEC E	PCR amplified gene	<i>E. coli</i> WA 837 genomic DNA
	PRLA-4	PCR amplified gene	<i>E. coli</i> prlA 4 gene from vector pRLA41++
	TRP P/O	Assembled synthetic oligonucleotide gene	<i>E. coli</i> tryptophan E promoter/operator Sequence from literature

* pelB - signal sequence of the pectate lyase B gene from *Erwinia carotovora*.

10 ++ The pRLA41 vector was provided by Tom Silhavy at Princeton University. The prlA-4 mutant allele may have properties that allow easier translocation of heterologous peptides and proteins. However, the natural prlA (secY) gene should function as well.

15 Transformation of *E. coli* BLR with pSCT-029A or pSCT-038

After constructing the pSCT-029A or pSCT-038 plasmids, i.e., ligation of the various DNA fragments, it is necessary to use the final ligation mixture to transform an *E. coli* host strain for propagation of the plasmid and for future protein expression work. To perform this transformation it is necessary to cause the *E. coli* cells to be competent to receive the DNA. The preparation of competent cells can be done by a variety of methods such as CaCl₂ treatment and electroporation.

25 For the final preparation of preferred cell lines, UGL 165 and UGL 703, we use both methods in series according to the following protocols.

I. Primary Transformation into the *E. coli* K-12 host, BB4

This first transformation is not essential, but is preferred because the *E. coli* BB4 K-12 host has a high transformation efficiency which results in a large number of transformants and a high probability of identifying a variety of desired clones.

A. Preparation of Competent BB4 Cells by CaCl_2 Treatment

BB4 Genotype...LE392.32 [F' $\text{lacI}^{\text{Q}}\Delta\text{MI5proAB Tn}$
10 (Tet^{R})]

- 10 1. Prepare an overnight saturated culture of the host cell.
2. Prepare a fresh host cell culture by inoculating 100 ml of medium to 0.5% (v/v) and grow to an A_{600} nm of 0.02-0.03.
- 15 3. Grow the culture to an A_{600} nm of 0.15-0.3, approximately 3 doublings.
4. Store the cells on ice for 10 minutes.
5. Remove the cells from the culture media by centrifugation, 5K rpm x 10 min.
- 20 6. Resuspend the pelleted cells in 0.5 volumes of ice cold 0.1 M CaCl_2 , store on ice for 30 minutes, then pellet the cells as previously described.
7. Resuspend the pelleted cells in 0.1X volume of 0.1 M CaCl_2 ; store on ice for 1 hour prior to use.
- 25

B. Transformation Protocol

1. To 100 ul of competent cells prepared as described add 1-2 ul of a ligation mixture which should ideally contain 2-10 ng of plasmid vector DNA.
- 30

2. Store the mixture on ice for 30 minutes.
3. Heat shock the mixture by placing in a heating block or water bath at 37°C.
4. Add to the mixture 1 ml of prewarmed culture medium and incubate the mixture at 37°C for 30-60 minutes.
5. Spread an appropriate amount of transformation mixture onto an appropriate solid media containing the necessary selective antibiotic and incubate the plates 18-24 hours until colonies appear.

II. Secondary transformation

Transformants are identified by a variety of methods. Several clones are chosen for transfer to the second host, *E. coli* B host strain, BLR. BLR is the host strain of choice for fermentation and protein expression.

The genotype of BLR is $F^- \text{ompT hsdS}_B(\text{r}_B \cdot \text{m}_B^-) \text{gal dcm } \Delta(\text{srl-recA}) 306::\text{Tn10}(\text{Tc}^R)$. *E. coli* cells will accept DNA after being exposed to an electric field under controlled and specified conditions. *E. coli* B host strains are more easily transformed with an intact plasmid, rather than a ligation mixture, and are more receptive to foreign DNA when made competent by electroporation than by CaCl_2 treatment.

A. Preparation of competent *E. coli* BLR cells for subsequent electroporation

1. Prepare an overnight saturated culture of the host cell.
2. Prepare a fresh host cell culture by inoculating 100ml of medium to 1.0% (v/v) and grow to an $A_{600 \text{ nm}}$ of 0.3-0.5.

3. Harvest the cells by centrifugation after chilling the culture on ice for 15 min.
4. Decant the supernatant, removing as much media as possible. Resuspend the cells in a total volume of 100 mls of ice cold aqueous 10% glycerol, w/v (the glycerol should be of extra high quality). Re-centrifuge the resuspended cells immediately.
5. Resuspend the cells in 50 mls of ice cold 10% glycerol (w/v). Re-centrifuge.
6. Resuspend the cells in 25 mls of ice cold 10% glycerol (w/v). Re-centrifuge.
7. Repeat step 6.
8. Resuspend the cells in a final volume of 2 mls of ice cold 10% glycerol (w/v). The final cell concentration should be $1-3 \times 10^{10}$ cells/ml. The cells can be stored for up to 1 year at -80°C .

B. Transformation by Electroporation

1. Incubate sterile cuvettes and the white chamber slide for 10 min on ice. Also incubate several polypropylene tubes on ice.
2. Mix 40 ul of cell suspension with 1-2 μl of solution containing plasmid DNA in Tris/EDTA at an approximate concentration of 100 pg/ μl .
(The DNA mixture must be as salt free as possible to prevent arcing of the equipment.) Mix the solution well and incubate on ice for 0.5-1.0 min.
3. Set the Gene-Pulser apparatus at 25 uF. Set the pulse controller resistance at 200 ohms. Set the Gene-Pulser apparatus to 2.5 Kv

with 0.2 cm cuvettes or 1.5-1.8 Kv when using
1.0 cm cuvettes.

Transfer the mixture of cells and DNA to a cold
electroporation cuvette and shake the
suspension to the bottom of the cuvette
removing all of the air bubbles. Place the
cuvette in the chilled safety chamber slide and
push the slide into the chamber until the
cuvette is seated between the contacts in the
base of the chamber.

4. Pulse the cuvette once at above setting.

5. Immediately add 1 ml of SOC (20 g Tryptone,
5 g yeast extract, 0.5 g NaCl, 1L H₂O and 20 mM
glucose) buffered media to the cuvette,
resuspend the cells.

6. Transfer the suspension to a sterile 17 x
100 mm polypropylene tube and incubate at 37°C
for 1 hour.

7. Plate the electroporated mixture on
selective media plates.

Production of sCTgly (Fermentation)

Fermentation batch media is prepared using the
components listed in Table 2. The fermentation is
inoculated with a late log phase culture grown in
inoculation media (Table 2). The inoculation volume is
determined by the amount of inoculum (number of cells)
needed to reach an initial A 600nm within the fermenter
between 0.015 and 0.24. The pH, DO₂, and temperature
parameters for the fermentation run are listed in
Table 3. The fed batch state of the fermentation is
started when the glycerol in the batch media is depleted.
[Glycerol depletion can be determined by a spike in

dissolved oxygen and/or a glycerol assay.] The feed rate is set to maintain a constant cell division rate based on cell mass (dry cell weight) at the time of glycerol depletion. Feed components are listed in Table 2. The feed rate is based on the following formula:

$$Q = \frac{(V) \times (MU) \times (dcw) \times (e^{Mu \cdot t})}{(Yx/sx[Feed])}$$

where:

Mu = growth rate (in doublings per hour)

dcw = dry cell weight in grams per liter of medium at feed start determined empirically for individual *E. coli* strains

t = time in hours

Y x/s = glycerol utilization constant for host (0.327 for *E. coli* WA 837; similar for BLR)

v = fermentation volume in liters

[feed] = grams glycerol per liter of feed medium (629 grams per liter used in all examples reported herein).

Q = glycerol feed rate, liters/hour

The induction process is accomplished using a gradient induction coupled to the feed rate increasing the inducer (IPTG) over time matching the set growth rate.

The fermentation in progress is monitored by measuring absorbance at 600nm, wet cell weight in g/l, in addition to CEX chromatography analysis of media samples for the presence and concentration of sCTgly in mg/l.

Glycine can be added to the fermentation in order to increase outer membrane permeability. Glycine can be added to the batch media or to the feed.

Preferably the glycine is added to the feed so that the glycine concentration in the fermentation culture increases with the rate of cell growth. The optimal concentration of glycine should be in the range of 0.1-1 grams per 100 ml at the final time point of the fermentation. In practice, the amount of glycine added to the feed is calculated so that the desired glycine concentration at the fermentation end is achieved. The actual amount of glycine added is dependent on the length of the fermentation in time, post induction, and final glycine concentration desired. The methods we have used: 24 g/l of glycine was added to the fermentation feed, which results in a final glycine concentration of 5 g/l at 26 hours post induction. We have found that adding glycine to the feed is more effective than adding glycine to the batch media.

Table 2. - Media Component List:

Inoculation Media

Components	Quantity g/L
(NH ₄) ₂ SO ₄	7.00
KH ₂ PO ₄	2.00
MgSO ₄ ·7H ₂ O	1.00
CaCl ₂	0.25
FeSO ₄ ·7H ₂ O	0.05
Sodium Citrate	1.50
N-Z Case +	5.00
Hy Yest 412	2.00
L-Methionine	4.50
Kanamycin	0.05
Glycerol	18.00

Batch Media

Components	Quantity g/L
(NH ₄) ₂ SO ₄	14.80
KH ₂ PO ₄	4.40
MgSO ₄ ·7H ₂ O	2.10
CaCl ₂	0.53
FeSO ₄ ·7H ₂ O	0.11
Sodium Citrate	2.20
N-Z Case +	10.60
Hy Yest 412	3.10
L-Methionine	4.50
Kanamycin	0.05
Glycerol	2.50

Feed Media

Components	Quantity g/L
Glycerol	629
IPTG	2.80

Table 3. - Conditions determined for fermentation parameters

Parameter	Most Preferred	Preferred	Good
pH	6.78-6.85	6.50-7.00	6.00-7.5
Temp °C	29.5-30.5	28.0-32.0	20.0-35.0
DO (oxygen saturation)	>80%	>70%	>50%
Mu value	0.12-0.14	0.10-0.16	0.05-0.20
Fermentation Time (Hours post induction)	24-27	22-29	20-32

10 Isolation of sCTgly

15 The conditioned medium is harvested by separating the cells from medium using either Tangential Flow Filtration or centrifugation to collect the media, and discarding the cells. The excreted sCTgly is stabilized in the media by adding 2.0 N HCl to a final pH of 3.0. The glycine-extended salmon calcitonin is stable for extended periods of time at pH 3.0. After cell removal and pH stabilization, the peptide is purified using cation exchange and reverse-phase chromatography methods. While reverse phase chromatography followed by cation exchange chromatography can provide good purification, it is preferred that an initial cation exchange step also be included prior to the reverse phase liquid chromatography. For large purification, this reduces the volume to be subjected to reverse phase chromatography, thus reducing environmental and safety concerns raised by the necessity of using high volumes of organic solvents such as acetonitrile.

Another preferred modification is S-sulfonation of the cysteine residues of the salmon calcitonin peptide prior to or during purification in order to improve yields of the monomeric peptide.

5 Description of clones

 The monogenic UGL 172 clone is an *E. coli* BLR host strain containing vector pSCT-025A which comprises one transcription cassette (monogenic) coding for salmon calcitonin with a C-terminal glycine (sCTgly).² The
10 digenic UGL 165 clone is an *E. coli* BLR host strain containing vector pSCT-029A which comprises two cassettes in tandem (digenic) each coding for salmon calcitonin with a C-terminal glycine (sCTgly). The trigenic UGL 168 clone is an *E. coli* BLR strain containing vector pSCT-034
15 which comprises three cassettes in tandem (trigenic) each coding for salmon calcitonin with a C-terminal glycine (sCTgly). The monogenic UGL 702 clone is an *E. coli* BLR strain containing vector pSCT-037 which comprises 1 cassette and secretion factor genes. The digenic UGL 703
20 clone is an *E. coli* BLR strain containing vector pSCT-038 which comprises 2 cassettes in tandem and secretion factor genes.

Example 1 - UGL 165 fermentation at 1L scale

25 The fermentation of the UGL 165 clone was carried out as described under Experimental details. Table 4 summarizes the fermentation parameters and results. Briefly, UGL

30 ² A preferred transcription cassette contains the dual tac/lac promoter followed by a ribosome binding site followed by sequences for the OmpA signal peptide followed by sequences for sCTgly followed by transcription terminator sequences rrnB T1-T2.

165 clonal cells were grown in inoculation medium and used to seed a fermenter containing 1 liter of batch medium to give an initial A_{600} nm of 0.06. Cells were grown for 6.25 hours until the glycerol in the medium was depleted. Then, the fed batch stage of the fermentation was started and supplemented continuously with the feed medium for 25.5 hours. The conditions at time zero (beginning of feed and induction) were as follows: oxygen saturation, 94%; temperature 30°C; and pH 6.8. The conditions at the end of fermentation (time 25.5 hours) were as follows: oxygen saturation, 40%; temperature 31°C; and pH 6.8. Also, at the end of fermentation, the absorbance at 600 nm was equal to 113.3 and the wet cell weight in g per liter was 168 g. The sCTgly production at the end of fermentation was also measured to be 222 mg/liter of medium (see figure 10 and table 4).

Table 4 - Fermentation Summary of UGL 165

Time point Hrs. Post Feed	A ₆₀₀ nm	wet cell weight g/L	sCT gly mg/L **	Feed vol added, mls	[IPTG] uM	Agitation RPM
-6.25	0.06	--	--	--	--	500
0***	11.1	--	--	--	--	1300
15	51.7	83.3	not assayed	44.1	528	1000
17	48.7	92.3	not assayed	51.3	614	1200
19	53.6	100.5	38	78.2	948	1300
21	67.6	117.3	77	104.8	1256	1300
22	86.7	130.9	111	120.4	1442	1350
23	92.6	140.0	104	138.2	1654	1350
24	106.5	153.7	156	158.4	1896	1350
25	108.5	162.8	183	181.2	2174	1400
25.5	113.3	168	222	194.1	2324	1400

** sCTgly determined using the CEX-HPLC assay as described in Example 4.

*** Agitation turned to 1450 rpm overnight @ 2 hrs post start feed

Example 2 -- UGL 703 fermentation at 1L scale

Recombinant *E. coli* UGL 703 has been deposited with the American Type Culture Collection (ATCC) as ATCC 98395 in accordance with the provision of the Budapest Treaty relating to the deposit of microorganisms for purposes of patent procedure. The fermentation of the UGL 703 clone was carried out as described under Experimental details. Table 5 summarizes the conditions of this fermentation. Briefly, UGL 703 clone was grown in inoculation medium and used to seed a fermenter containing 1 liter batch medium to give an initial A₆₀₀ nm of 0.06 (preference is 0.06 to 0.12). Cells were grown for 6.25 hours (preferred range is from 6.0 to 7.0 hours) until the

glycerol in the medium was depleted. Then, the fed batch stage of the fermentation was started and supplemented continuously with the feed medium for 26 hours. The conditions at time zero (beginning of feed and induction) were as follows: oxygen saturation, 95%; temperature 30°C; and pH 6.8. The conditions at the end of fermentation (time 26 hours) were as follows: oxygen saturation, 80%; temperature 31°C; and pH 6.8. Also, at the end of fermentation, the absorbance at 600 nm was equal to 80.9 and the wet cell weight in grams per liter was 129.1. The sCTgly production was also measured to be 284 mg/liter of medium (see figure 11 and table 5).

Table 5 - Fermentation Summary of UGL 703

Time point	A 600	wet cell weight g/L	sCT gly mg/L **	Feed vol added, mls	[IPTG] uM	Agitation RPM
-6.5	0.06	--	--	--	--	500
0	8.1	--	--	--	--	1300
15.5	22.6	7.3	~18	47.5	570	1150
19	36.5	83.1	25	72.9	948	1250
21	53.5	94.2	53	104.8	1256	1350
22	61.3	100.8	58	120.4	1442	1450
23	59.7	106.0	69	138.2	1654	1550
24	73.3	119.0	83	158.4	1898	1580
25	74.1	119.1	203	181.1	2174	1620
26	80.9	129.1	284	207.6	2487	1620

** sCTgly determined using the CEX-HPLC assay as described in Example 4.

Conclusions

Other experiments were carried out under similar overall conditions using UGL 172, UGL 168 and UGL 702 clones. Figures 12 and 13 indicate that the digenic UGL 165 clone is best suited for production of sCTgly

with the trigenic UGL 165 clone being second best over the monogenic UGL 173 clone. However, the production of sCTgly by UGL 165 can still be improved in the presence of co-expressed secretion factors (UGL 703) (compare Figures 10 and 11 and 17).

With regard to oxygen saturation during fermentation, Figures 14A and 14B support the conclusion that added oxygen in the fermentation medium is not critical to cell growth of UGL 165 but is very important in increasing the production of sCTgly.

Figures 15A, 15B, and 16 clearly indicate that the *E. coli* strain BLR is best suited for production of sCTgly.

The production of sCTgly can be still further increased by the addition of glycine as an added feed component.

Example 3 - Purification of sCTgly from UGL 165 Culture Media: Cation-Exchange Chromatography #1:

Approximately 1000 L of culture media which had been harvested by either tangential flow filtration or centrifugation was acidified with a sufficient volume of 2N hydrochloric acid to decrease the pH to 3.0. The media was subsequently diluted with a sufficient volume of water to decrease the conductivity to ≤ 7.5 mS. The diluted media was loaded onto a cation-exchange column (Pharmacia SP-Sepharose Big Beads, 99.0 cm x 13.0 cm) which had been equilibrated with 10 mM citric acid pH 3.0 at a flow rate of 25 L/min. (3.25 cm/min.). After the loading was complete, the column was washed with 10 mM citric acid pH 3.0 at 8 L/min (1.0 cm/min) for approximately 40 minutes (3 bed volumes) or until a stable UV baseline was achieved. The product (sCTgly)

was eluted with 10 mM citric acid, 350 mM sodium chloride
pH 3.0 at a flow rate of 8 L/min. (1.0 cm/min.). The
column was cleaned and sanitized with 0.5 M sodium
hydroxide. 1.0 M sodium chloride for 60.0 minutes (5.0
5 bed volumes) at 8 L/min. (1.0 cm/min).

To a stirred tank containing the resulting
CEX#1 eluate (approximately 100 L) is added 60.57 grams
of tromethamine. The solution is stirred until all
solids are dissolved. The pH of the solution is adjusted
10 to 8.25 [range: 8.0 to 8.5] using 2 M NaOH. 23.64 grams
of TRIS•HCl (Tris[hydroxymethyl]aminomethane
hydrochloride) is added and the solution is stirred until
all solids are dissolved. A solution of 1.0 kg of sodium
sulfite dissolved in TRIS•HCl and a solution of 200 grams
15 of sodium tetrathionate dissolved in TRIS•HCl are added
to the tank with stirring. The reaction is allowed to
stir for 15 minutes. If necessary, the pH is adjusted to
8.25 [range: 8.0 to 8.5] with 2 M NaOH. The reaction
mixture is stirred overnight at room temperature. The pH
20 of the reaction mixture is adjusted to 2.25 [range: 2.0
to 2.5] with 2 M HCl.

Reverse-Phase Chromatography #1 (RP #1):

The resulting S-sulfonation reaction mixture
(approximately 100 L) was loaded directly onto a reverse-
25 phase column (Toso Haas Amberchrom CG300md, 25.0 cm x
18.0 cm) which had been equilibrated with 0.1%
trifluoroacetic acid at 2.0 L/min. (4.0 cm/min). After
loading was complete, the column was washed with 0.1%
trifluoroacetic acid at 750 ml/min. (1.5 cm/min.) until a
30 stable UV baseline was achieved. The column was washed
with 0.1% trifluoroacetic acid, 20% acetonitrile at 750
ml/min. (1.5 cm/min.) until the principal contaminant

peak completely eluted. The product (sCTgly) was eluted with 0.1% trifluoroacetic acid, 40% acetonitrile at 750 ml/min. (1.5 cm/min.). The column was cleaned with 0.1% trifluoroacetic acid, 80% acetonitrile for 30 minutes at 750 ml/min. (1.5 cm/min.).

Cation-Exchange Chromatography #2 (CEX #2):

The resulting RP #1 eluate (approximately 8.0 L) was loaded directly onto a cation-exchange column (E. Merck Fractogel EMD SO3 650M, 18.0 cm x 24.0 cm) which had been equilibrated with 25 mM MES (2-[N-morpholino]-ethanesulfonic acid) pH 5.8 at 500 ml/min. (2.0 cm/min.). After the loading was complete, the column was washed at 750 ml/min. (3.0 cm/min.) with 25 mM MES (2-[N-morpholino]-ethanesulfonic acid) pH 5.8 until the column effluent returned to pH 5.8 (range 5.6-5.9). The column was washed with 25 mM MES (2-[N-morpholino]-ethanesulfonic acid), pH 5.8 for an additional 30 minutes at 750 ml/min. (3.0 m/min.) to remove the principal peptide contaminants. The product (sCTgly) was eluted with 25 mM MES, 100 mM sodium chloride pH 5.8 at 750 ml/min. (3.0 cm/min.). The product fraction is adjusted to pH 3.0 - 5.0 with 1.0 M HCl unless amidation follows immediately. The column was cleaned and sanitized with 0.1 M sodium hydroxide, 1.0 M sodium chloride for 60 minutes at 750 ml/min. (3.0 m/min.).

Amidation Reaction.

The resulting pH-adjusted CEX #2 eluate contains purified sCTgly which is a suitable substrate solution for use in the *in vitro* conversion of sCTgly to authentic salmon calcitonin, a reaction catalyzed by

peptidyl glycine α -amidating enzyme (PAM) as shown below in Example 5.

Example 4

Analytical Cation-Exchange HPLC for quantification of sCTgly:

sCTgly in collected chromatography fractions was identified and quantified by analytical CEX-HPLC. An aliquot of each fraction was loaded onto a cation-exchange column (The Nest Group, Polysulfoethyl aspartamide, 4.6 mm x 50 mm) which had been equilibrated with 10 mM sodium phosphate pH 5.0 at a flow rate of 1.2 ml/min. Separation was achieved by performing a linear gradient from 10 mM sodium phosphate pH 5.0 to 10 mM sodium phosphate, 250 mM sodium chloride pH 5.0 at 1.2 ml/min over 15 minutes. The column effluent was monitored by UV absorbance at 220 nm. sCTgly was identified by comparison of its retention time to that of a purified sCTgly reference standard. sCTgly was quantified by peak area as compared to the sCTgly reference standard. This analytical method was also used to quantify SCTgly from the fermentation medium.

Example 5 - Conversion of Glycine-extended salmon calcitonin to authentic salmon calcitonin using α -amidating enzyme

In order to obtain the optimal yields of amidated salmon calcitonin, the following critical parameters are observed:

- 1) The amidation reaction is carried out in a silanized glass vessel to prevent non-specific adsorption of peptide to the reaction vessel.

2) A high level of dissolved oxygen is maintained in the reaction mixture by sparging and/or agitation. Preferably, the level of dissolved oxygen is $\geq 75\%$.

5 3) Incubation temperature during amidation is maintained between 35°C - 39°C.

4) The pH of the amidation reaction is maintained between 6.0 and 6.5.

10 5) The starting concentration of glycine-extended salmon calcitonin in the amidation reaction should be between 3.5 - 10.5 mg/ml (0.95 mM to 2.9 mM).

15 6) When 12,000 - 24,000 units/ml of substantially protease-free α -amidating enzyme (peptidyl glycine α -amidating monooxygenase, herein referred to as "PAM") are added to the reaction mixture and the concentration of substrate is as indicated in 5) above, the reaction is allowed to proceed for 4-6 hours. However, the reaction time can be further increased up to 24 hours without deleterious effects to the product.

20 7) To prevent the amidation reaction from becoming ascorbate limiting, an additional equivalent of ascorbate is added at about the midpoint of the reaction.

The components of the amidation reaction mixture are the following:

25 3.5 - 10.5 mg/ml of S-sulfonated, glycine extended salmon calcitonin

30 mM MES buffer, pH 6.0 - 6.5

0.5 to 1.0 uM CuSO₄ (e.g. 0.5)

4-15 mM KI (e.g. 5)

30 1-5% Ethanol (e.g. 1%)

10-100 ug/ml Catalase (e.g. 35)

1.5-3.0 mM Ascorbate (e.g. 1.5)

peptidyl glycine α -amidating monooxygenase
(12,000 - 24,000 units per ml of reaction mixture. 1
unit is 1 picomole per minute conversion of DansylTyr-
Val-Gly substrate to product at 37°C at pH 7). The PAM
5 enzyme may be obtained as described in Miller et al., ABB
298: 380-388 (1992) U.S. Patent 4,708,934, European
publication 0 308 067 and 0 382 403, and Biotechnology
Vol. II (1993) pp. 64-70, the disclosures of which are
hereby incorporated by reference.

10 The glycine extended salmon calcitonin may be
produced by the fermentation as described in Example 1 or
Example 2 and purified as described in Example 3 prior to
amidation.

In instances where the enzyme used for
15 amidation is peptidyl glycine α -hydroxylating
monooxygenase (PHM), the same reaction mixture is used as
that described above, substituting PHM for PAM. In
addition, at the end of the 4 to 6 hour incubation
period, the pH of the reaction mixture is increased by
20 the addition of base to between 8 and 9. The reaction
mixture is agitated for an additional 4 to 8 hours prior
to terminating the reaction. Peptidyl glycine α -
hydroxylating monooxygenase may be obtained by expressing
only the N-terminal portion of PAM (about the first 40
25 dKa). See e.g. Mizuno et al. BBRC Vol. 148, No. 2, pp.
546-52 (1987) the disclosure of which (as it relates to
Mizuno's "AE 1" is incorporated herein by reference.
Frog skin is known to express PHM naturally.

After the amidation reaction has been
30 terminated, the reaction is diluted with sufficient water
to bring the final peptide concentration to less than 3.0
mg/ml. Sufficient 1 M TRIS pH 9.0 is added to the
mixture to bring the final concentration of TRIS to

approximately 100 mM. If necessary, the pH is adjusted to [8.0 to 9.0] with 2 M NaOH. A 3.0 fold excess of L-cysteine, over the final concentration (mM) of SO₃-SCT, is added slowly with stirring to the reaction mixture.

5 If necessary, the pH is adjusted to [8.0 to 8.5] with 2 M NaOH. The renaturation reaction is stirred for 1 hour at room temperature. The reaction is terminated by acidification with 10% phosphoric acid to pH 2.0 [1.9 to 2.3].

10 **Example 6**

Post- Amidation Purification

Cation exchange chromatography #3 (CEX #3): This column is used to purify SCT following α -amidation and renaturation. The principal contaminant following α -amidation/renaturation is SCTG. CEX #3 chromatography
15 employs an Amicon Vantage-A column (18.0 x 16.0 cm) packed with Toyopearl SP650S resin. The unit operation is accomplished using water for injection (WFI) and solutions of 0.5 M, 50 mM and 175 mM sodium chloride
20 along with 150 mM sodium phosphate pH 5.5. A brief description of the process steps follows:

1) The operational flow rate is set to 750 ml/min.

2) The following parameters which are used to
25 monitor the chromatography are set using LC system controller:

UV wavelength 230 nm

Range 0.64 AUFS

Conductivity X1000

30 3) The column is initially washed with WFI for at least 5 minutes at a flow rate of 750 ml/min.

4) The dilution pump (150 mM sodium phosphate pH 5.0) is set to 50 ml/min. and the column is equilibrated with 10 mM sodium phosphate at a flow rate of 750 ml/min until a stable pH baseline is observed.

5) The column is re-equilibrated with 10 mM sodium phosphate at a flow rate of 750 ml/min until a stable pH baseline below 6.0 is achieved. (Note: If the pH of the column is not below 6.0 then a 150 mM sodium phosphate wash is required.) If the 150 mM sodium phosphate pH 5.5 wash is performed the column must be re-equilibrated using 10 mM sodium phosphate pH 5.5 before proceeding at the next step.

6) Following the re-equilibration, the column is subjected to a blank elution with 175 mM sodium chloride; 10 mM sodium phosphate pH 5.5 for 4 minutes at a flow rate of 750 ml/min.

7) The column is re-equilibrated with 10 mM sodium phosphate pH 5.5 at a flow rate of 750 ml/min until a stable pH baseline is achieved.

8) Once equilibration is achieved the amidated/renatured output containing 10-25 gram of SCT is pH adjusted to 3.5 using 2 N sodium hydroxide and loaded onto the CEX #3 column at 400 ml/min. The sample load is chased by rinsing the load container with 500 ml of WFI.

9) Following the load the column is washed with 10 mM sodium phosphate pH 5.5 at a flow rate of 750 ml/min. for 30 minutes or until the pH of the column stabilizes above 5.0.

10) Once the pH of the column has stabilized above 5.0 the column is washed, at 750 ml/min., with 50 mM sodium chloride; 10 mM sodium phosphate pH 5.5 for 100 minutes or until the SCTgly peak emerges.

11) Once the 100 minutes has expired the 175 mM sodium chloride is attached to the system. The column is washed with 175 mM sodium chloride; 10 mM sodium phosphate pH 5.5 at a flow rate of 750 ml/min. and the product eluted. The entire product peak is collected in one container. The weight of the CEX3 output material is determined and 1 N acetic acid (10% fraction weight) is added to the fraction.

12) The column is stripped with 0.5 M sodium chloride; 10 mM sodium phosphate pH 5.5 at a flow rate of 750 ml/min. for 15 minutes.

13) Once the column has been stripped with 1.0 M sodium chloride/0.25 N sodium hydroxide is attached to the system. The dilution pump is set to 0.000 ml/min. and the column washed with 1.0 M sodium chloride/0.25 N sodium hydroxide at a flow rate of 600 ml/min. for at least 30 minutes.

14) The column is washed with WFI for 5 minutes at a flow rate of 750 ml/min.

15) The column is washed with 10 mM sodium hydroxide at a flow rate of 500 ml/min. for at 30 minutes. The column is stored under these conditions.

Reverse-phase chromatography (RP #2): This step follows CEX #3 and is used as a salt and buffer exchange step prior to lyophilization. The main objective of the step is to exchange the salt with acetate. RP #2 chromatography employs an Amicon Vantage-A column (13.0 x 12.5 cm) packed with Amberchrom CG300md resin. The unit operation is accomplished using water for injection, ethyl alcohol, 250 mM sodium acetate and 0.5% acetic acid. A brief description of the process steps follows:

1) The CEX #3 eluate (approximately 4 liters) is acidified to pH 2.0 with phosphoric acid and then diluted with 3 equal volumes of 333 mM sodium acetate solution, and allowed to stand for at least 1 hour.

5 2) The flow rate is set to 320 ml/min. while the dilution pump (0.5% acetic acid) is set to 80 ml/min. for an overall operational flow rate of 400 ml/min.

 3) The following parameters which are used to monitor the chromatography are set using LC system controller:

 UV wavelength 230 nm

 Range 2.54 AUFS

 Conductivity X 1000

15 4) The column is initially washed with 0.1% acetic acid at a flow rate of 400 ml/min. until a stable conductivity baseline is observed.

 5) The column is stripped with 80% ethyl alcohol, 0.1% acetic acid at a flow rate of 400 ml/min. until a stable pH baseline is observed.

20 6) The column is washed with 0.1% acetic acid until a stable pH baseline is observed.

 7) Following the wash, a column test is performed to track resin cleaning. The column is subjected to a blank elution with 40% ethyl alcohol; 0.1% acetic acid for 6 minutes at a flow rate of 400 ml/min. The collected eluate from the column test is submitted at QC for analytical testing.

25 8) The column is washed with 0.1% acetic acid at a flow rate of 400 ml/min until a stable conductivity baseline is observed.

30 9) Upon completion of the wash, the WFI is disconnected from the inlet and the 250 mM sodium acetate is connected. The dilution pump is set to 0.000 ml/min.

The column is equilibrated with 250 mM sodium acetate at a flow rate of 400 ml/min. until a stable pH baseline is observed.

5 10) Once equilibration is achieved, the CEX #3 eluate is loaded onto the RP #2 column at 400 ml/min. The sample load is chased by rinsing the load container with 1.0 liters of 250 mM sodium acetate.

 11) The column is washed using 250 mM sodium acetate at a flow rate of 400 ml/min. for 60 minutes.

10 12) Following the sodium acetate wash, the sodium acetate is disconnected from the inlet and the WFI is connected. The dilution pump is returned to a flow rate of 80 ml/min. and the column is washed with 0.1% acetic acid at a flow rate of 400 ml/min for 25 minutes.

15 13) Following the 0.1% acetic acid wash, the product is eluted using 40% ethyl alcohol; 0.1% acetic acid at a flow rate of 400 ml/min. The entire product peak is collected, and subjected to lyophilization to yield purified SCT powder.

20 14) The column is stripped with 80% ethyl alcohol; 0.1% acetic acid at a flow rate of 400 ml/min. for at least 20 minutes.

 15) Following the strip, the dilution pump is set to 0.000 ml/min. and the column is washed with WFI at a flow rate of 400 ml/min. for at least 5 minutes.

25 16) After the WFI wash, the WFI is disconnected from the inlet and the 0.5N sodium hydroxide is connected. The column is washed with the 0.5N sodium hydroxide at a flow rate of 400 ml/min. for at least 20 minutes.

30 17) The 0.5N sodium hydroxide is disconnected from the system and the WFI is connected. The column is washed with 50% ethyl alcohol at a flow rate of 400

ml/min. for at least 20 minutes. The column is stored under these conditions.

18) The RP #3 eluate is stored at 2 to 8°C.

Although the present invention has been
5 described in relation to particular embodiments thereof,
many other variations and modifications and other uses
will become apparent to those skilled in the art. The
present invention therefore is not limited by the
specific disclosure herein, but only by the appended
10 claims.

WHAT IS CLAIMED IS:

1. An expression vector comprising:
 - (a) a coding region with nucleic acids coding for a peptide product coupled in reading frame 3' of nucleic acids coding for a signal peptide; and
 - 5 (b) a control region linked operably with the coding region, said control region comprising a plurality of promoters and at least one ribosome binding site, wherein at least one of said promoters is tac.
2. The vector of claim 1, comprising a plurality of transcription cassettes, each cassette having said control region and said coding region.
3. The vector of claim 1, further comprising nucleic acids coding for a repressor peptide capable of repressing expression controlled by at least one of said promoters.
4. The vector of claim 3, wherein the nucleic acids coding for the repressor encode a lac repressor.
5. The vector of claim 1, wherein said control region has exactly two promoters.
6. The vector of claim 1, wherein said tac promoter is 5' of another promoter in said control region.
7. The vector of claim 1, wherein the control region comprises both a tac promoter and a lac promoter.

8. The vector of claim 7, wherein the lac promoter is 3' of the tac promoter.

9. The vector of claim 1, wherein said nucleic acids coding for the signal peptide encode a signal peptide for secreted bacterial proteins.

10. The vector of claim 9, wherein said signal is OmpA signal peptide.

11. The vector of claim 1, wherein said peptide product has a molecular weight of less than 10 KDa.

12. The vector of claim 1, wherein the C-terminal amino acid of said peptide product is glycine.

13. The vector of claim 12, wherein said peptide product is salmon calcitonin precursor.

14. The vector of claim 12, wherein said peptide product is calcitonin gene related peptide precursor.

15. The vector of claim 12, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone, and a 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

16. The vector of claim 1, further comprising nucleic acids coding for at least one secretion enhancing peptide.

17. The vector of claim 16, wherein the secretion enhancing peptide is selected from the group consisting of secY and prlA-4.

18. The vector of claim 16, wherein the secretion enhancing peptide is secE.

19. The vector of claim 16, wherein a plurality of secretion enhancing peptides are encoded, at least one of which is secE and the other of which is selected from the group consisting of secY and prlA-4.

20. A host cell transformed or transfected with the vector of claim 1.

21. A host cell transformed or transfected with the vector of claim 16.

22. The host cell of claim 20, wherein said host cell is a bacterial cell.

23. The host cell of claim 22, wherein said bacterial cell is a gram negative bacterial cell.

24. The host cell of claim 22, wherein said bacterial cell is *E. coli*.

25. The host cell of claim 24, wherein said *E. coli* is strain BLR.

26. The host cell of claim 24, wherein said *E. coli* is strain BL21.

27. The host cell of claim 24, wherein said *E. coli* is strain WA837.

28. The host cell of claim 22, wherein said host cell further expresses at least one secretion-enhancing peptide.

29. The host cell of claim 28, wherein the secretion enhancing peptide is selected from the group consisting of *secY* and *prlA-4*.

30. The host cell of claim 28, wherein the secretion enhancing peptide is *secE*.

31. The host cell of claim 28, wherein a plurality of secretion enhancing peptides are encoded, at least one of which is *secE* and the other of which is selected from the group consisting of *secY* and *prlA-4*.

32. An *E. coli* host containing and expressing an expression vector which comprises a plurality of transcription cassettes in tandem, each cassette comprising:

(a) a coding region comprising nucleic acids coding for a peptide product coupled in reading frame 3' of nucleic acids coding for a signal peptide; and

(b) a control region linked operably with the coding region, said control region comprising a plurality of promoters in tandem and at least one ribosome binding

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site, wherein at least one of said promoters is selected from the group consisting of tac and lac.

33. The host of claim 32, wherein said control region comprises from 5' to 3' a tac promoter and a lac promoter.

34. The host of claim 32, wherein said vector has exactly two transcription cassettes in tandem.

35. A host cell transformed with an expression vector which comprises a gene for expressing salmon calcitonin precursor, said host cell being *E. coli* strain BLR.

36. A host cell transformed with an expression vector which comprises a gene for expressing calcitonin gene related peptide precursor, said host being *E. coli* strain BLR.

37. A method of producing a peptide product which comprises culturing the host cell of claim 20 in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

38. The method of claim 37 wherein peptide product yield exceeds 100 mg per liter of media.

39. A method of producing a peptide product which comprises culturing the host cell of claim 21 in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

40. A method of producing a peptide product which comprises culturing the host cell of claim 35 in a culture medium and then recovering the peptide product from the medium in which the host cell has been cultured.

41. The method of claim 37, wherein the peptide product is salmon calcitonin precursor.

42. The method of claim 37, wherein the peptide product is calcitonin gene related peptide precursor.

43. The vector of claim 1, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone and 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

44. The method of claim 38, wherein a method of induction is started prior to stationary phase.

45. The method of claim 44, wherein the induction method is by addition of a chemical inducer.

46. The method of claim 45, wherein the induction is by addition of at least one inducer selected from the group consisting of IPTG and lactose.

47. A method of producing a peptide product which comprises the steps of:

(a) culturing host cells of claim 20 in a culture medium in the presence of a carbon source and

inducing the expression of the peptide product, while controlling growth of said host cells at a growth rate between 0.05 and 0.20 doublings per hour; and

5 (b) thereafter recovering said peptide product from the medium.

48. The method of claim 47, wherein a membrane-permeabilizing amount of glycine is present in the medium during at least a portion of said controlled growth.

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49. The method of claim 47, wherein an inducer and carbon source are added during each hour of culturing in amounts such that the weight ratio of the inducer to the carbon source added in any one hour does not vary by more than 50% from the ratio added during the entire fermentation period.

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50. The method of claim 47, wherein no external carbon source is introduced into the medium until carbon source initially present in said medium is depleted to a level at which it could not continue to support the life of said host absent introduction of external carbon source into the medium, and wherein carbon source is thereafter added at a rate which maintains said growth rate between 0.05 and 0.20 doublings per hour.

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51. The method of claim 47, wherein the host cell is cultured for a period between 20 and 32 hours post induction.

52. The method of claim 47, wherein the host cell is cultured for a period between 24 and 27 hours post induction.

53. The method of claim 47, wherein the host cell is cultured at a temperature between 20 and 35°C.

54. The method of claim 47, wherein the host cell is cultured at a temperature between 28 and 32°C.

55. The method of claim 47, wherein the host cell is cultured at a temperature between 29.5 and 30.5°C.

56. The method of claim 47, wherein the pH of the culture medium is between 6.0 and 7.5.

57. The method of claim 47, wherein the pH of the culture medium is between 6.78 and 6.85.

58. The method of claim 47, wherein the pH of the culture medium is between 6.6 and 7.0.

59. The method of claim 47, wherein oxygen saturation of the culture medium is at least 20%.

60. The method of claim 47, wherein oxygen saturation of the culture medium is at least 50%.

61. The method of claim 47, wherein the oxygen saturation of the culture medium is at least 80%.

62. The method of claim 46, wherein the average cell growth rate during the culture period is maintained between 0.10 and 0.15 doublings per hour.

63. The method of claim 55, wherein the growth rate is maintained at about 0.13 doublings per hour.

64. The method of claim 49, wherein the carbon source is glycerol.

65. The method of claim 37, wherein said peptide product is salmon calcitonin precursor.

66. The method of claim 37, wherein said peptide product is calcitonin gene related peptide precursor.

67. The method of claim 37, wherein said peptide product is selected from the group consisting of parathyroid hormone, the first 34 amino acids of parathyroid hormone and 35 amino acid peptide having a C-terminal glycine in position 35 and the first 34 amino acids of parathyroid hormone in positions 1-34.

68. The method of claim 47, wherein induction is achieved by use of a chemical inducer.

69. The method of claim 58, wherein the inducer is at least one agent selected from the group consisting of lactose and IPTG.

70. The method of claim 37, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

5 (b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

(c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

71. The method of claim 39, wherein recovering said peptide product comprises:

5 (a) separating host cells from the culture medium; and

(b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

10 (c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

72. The method of claim 47, wherein recovering said peptide product comprises:

5 (a) separating host cells from the culture medium; and

(b) subjecting the medium to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

10 (c) subjecting said fractions of step (b) to cation exchange chromatography, and

(d) thereafter recovering fractions containing peptide product.

73. The method of claim 37, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

5 (b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

10 (c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product;

(d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

74. The method of claim 39, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

5 (b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

10 (c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product;

(d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

75. The method of claim 47, wherein recovering said peptide product comprises:

(a) separating host cells from the culture medium; and

5 (b) subjecting the medium to cation exchange chromatography and recovering fractions containing said peptide product; and

(c) subjecting the recovered fraction of step (b) to reverse-phase liquid chromatography and recovering fractions containing peptide product; and

10 (d) subjecting the recovered fractions of step (c) to cation exchange chromatography, and

(e) thereafter recovering fractions containing peptide product.

5 76. The method of claim 70, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

5 77. The method of claim 71, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

5 78. The method of claim 72, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

5 79. The method of claim 73, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

5 80. The method of claim 74, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

5 81. The method of claim 75, wherein the peptide product has at least one cysteine in its molecular structure and wherein at least one sulfhydryl group of a cysteine of the peptide product is sulfonated during at least a portion of said method of recovering peptide product.

82. The method of claim 72, further comprising altering the pH of the culture medium, immediately after terminating fermentation, to a level where proteolytic degradation of product is reduced.

83. The method of claim 82, wherein salmon calcitonin precursor is the peptide product and pH is adjusted to between 2.5 and 4.0.

84. The method of claim 83, wherein the pH is adjusted to between 3 and 3.5.

85. The method of claim 82, further comprising lowering the temperature of the culture medium to below 10°C after fermentation is terminated.

86. A method of producing an amidated peptide product comprising the steps of:

(a) culturing the host cell of claim 20 in a culture medium wherein the peptide product includes a C-terminal glycine;

(b) recovering said peptide product from said culture medium; and

(c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

87. A method of producing an amidated peptide product comprising the steps of:

(a) culturing the host cell of claim 35 in a culture medium wherein the peptide product includes a C-terminal glycine;

(b) recovering said peptide product from said culture medium; and

(c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

88. A method of producing an amidated peptide comprising:

(a) culturing host cells which express a peptide product having a C-terminal glycine together with an N-terminal signal peptide under conditions wherein growth of said host cells is controlled to stay within a range of 0.05 to 0.20 doublings per hour; wherein the

culture is induced during some of the period of said controlled growth;

(b) recovering said peptide product from the culture media; and

5 (c) converting said peptide product to an amidated peptide by converting said C-terminal glycine to an amino group.

89. The method of claim 83, wherein the host cell is cultured in a culture medium in the presence of an inducer, while maintaining an average cell growth rate during culturing between 0.05 and 0.20 doublings per hour.

90. The method of claim 84, wherein the host cell is cultured in a culture medium in the presence of an inducer, while maintaining an average cell growth rate during culturing between 0.05 and 0.20 doublings per hour.

91. The method of claim 86, wherein the peptide product is salmon calcitonin precursor or calcitonin gene related peptide precursor.

92. The method of claim 87, wherein the peptide product is salmon calcitonin precursor or calcitonin gene related peptide precursor.

93. The method of claim 86, wherein said conversion to amidated peptide is accomplished by:

(a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine α -amidating

monooxygenase, or peptidyl glycine α -hydroxylating monooxygenase;

(b) if peptidyl glycine α -amidating monooxygenase is not used in step (a), and if the reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic; and

(c) recovering said amidated peptide from said reaction mixture.

94. The method of claim 87, wherein said conversion to amidated peptide is accomplished by:

(a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine α -amidating monooxygenase, or peptidyl glycine α -hydroxylating monooxygenase;

(b) if peptidyl glycine α -amidating monooxygenase is not used in step (a), and if the reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic; and

(c) recovering said amidated peptide from said reaction mixture.

95. The method of claim 88, wherein said conversion to amidated peptide is accomplished by:

(a) forming a reaction mixture by contacting said peptide product with oxygen and a reducing agent in the presence of peptidyl glycine α -amidating monooxygenase, or peptidyl glycine α -hydroxylating monooxygenase;

(b) if peptidyl glycine α -amidating monooxygenase is not used in step (a), and if the

reaction mixture is not already basic, then increasing pH of the reaction mixture until it is basic; and

(c) recovering said amidated peptide from said reaction mixture.

96. The method of claim 93, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

97. The method of claim 94, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

98. The method of claim 84, wherein recovering amidated peptide comprises at least one of the steps selected from the group consisting of cation exchange chromatography and reverse phase chromatography.

99. A method for direct expression of a peptide product into a culture medium comprising the steps of:

(a) culturing host cells which express said peptide product together with a signal peptide, in said medium, under conditions wherein growth of said host cells is controlled to stay within a range of 0.05 to 0.20 doublings per hour; wherein an inducer is present during some of said period of controlled growth; and

(b) recovering said peptide product from the culture medium.

101. The method of claim 99 wherein oxygen saturation averages over 50% in said medium during the period of controlled growth.

P/546-192

DIRECT EXPRESSION OF PEPTIDES INTO CULTURE MEDIA

ABSTRACT OF THE DISCLOSURE

Expression systems are disclosed for the direct expression of peptide products into the culture media where genetically engineered host cells are grown. High yield was achieved with novel vectors, a special selection of hosts, and/or fermentation processes which include careful control of cell growth rate, and use of an inducer during growth phase. Special vectors are provided which include control regions having multiple promoters linked operably with coding regions encoding a signal peptide upstream from a coding region encoding the peptide of interest. Multiple transcription cassettes are also used to increase yield. The production of amidated peptides using the expression systems is also disclosed.

Construction of pSCT-018D

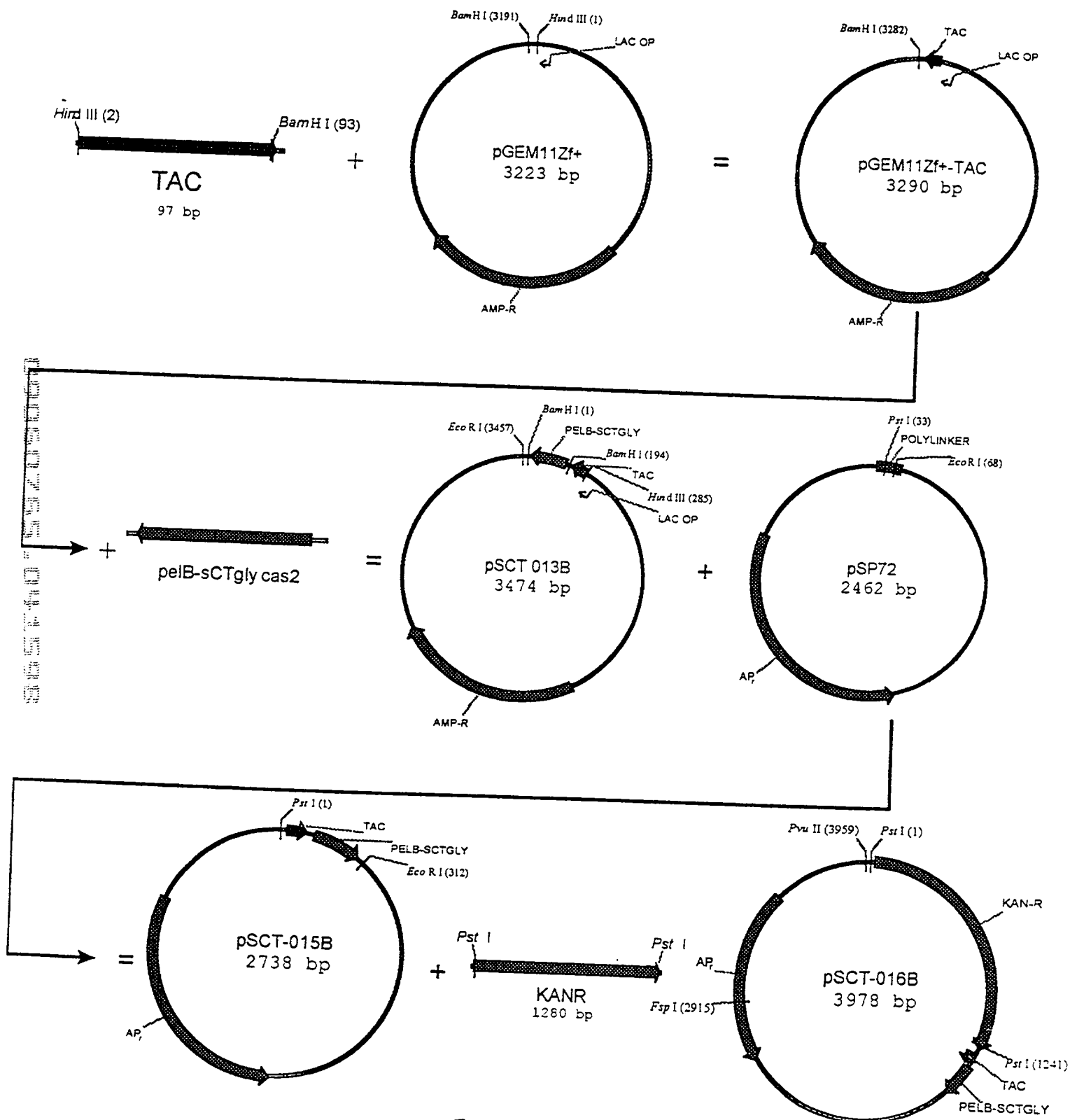


FIGURE 1A

Construction of pSCT-018D cont

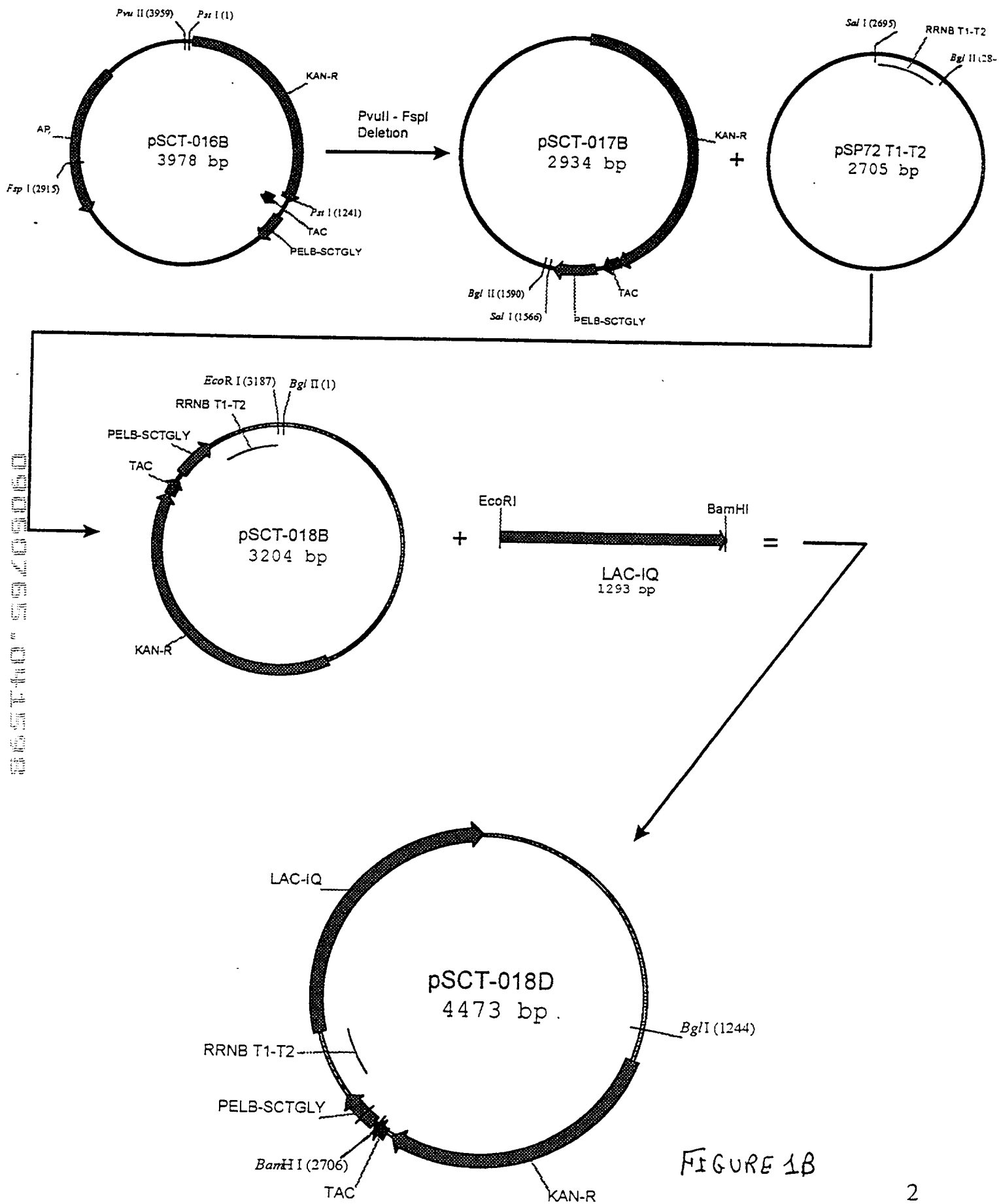


FIGURE 1B

Construction of pSCT-019

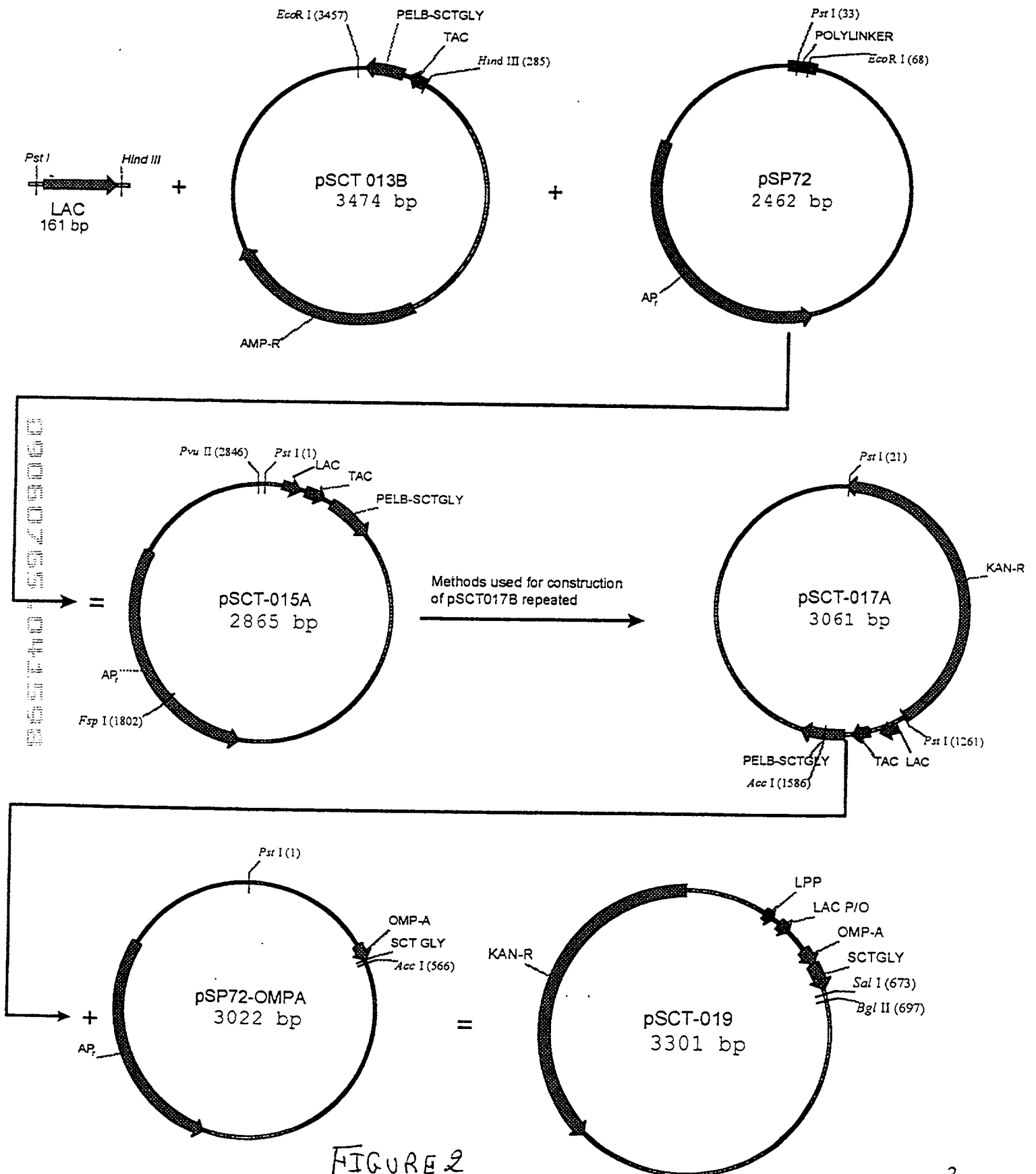


FIGURE 2

Construction of pSCT-025

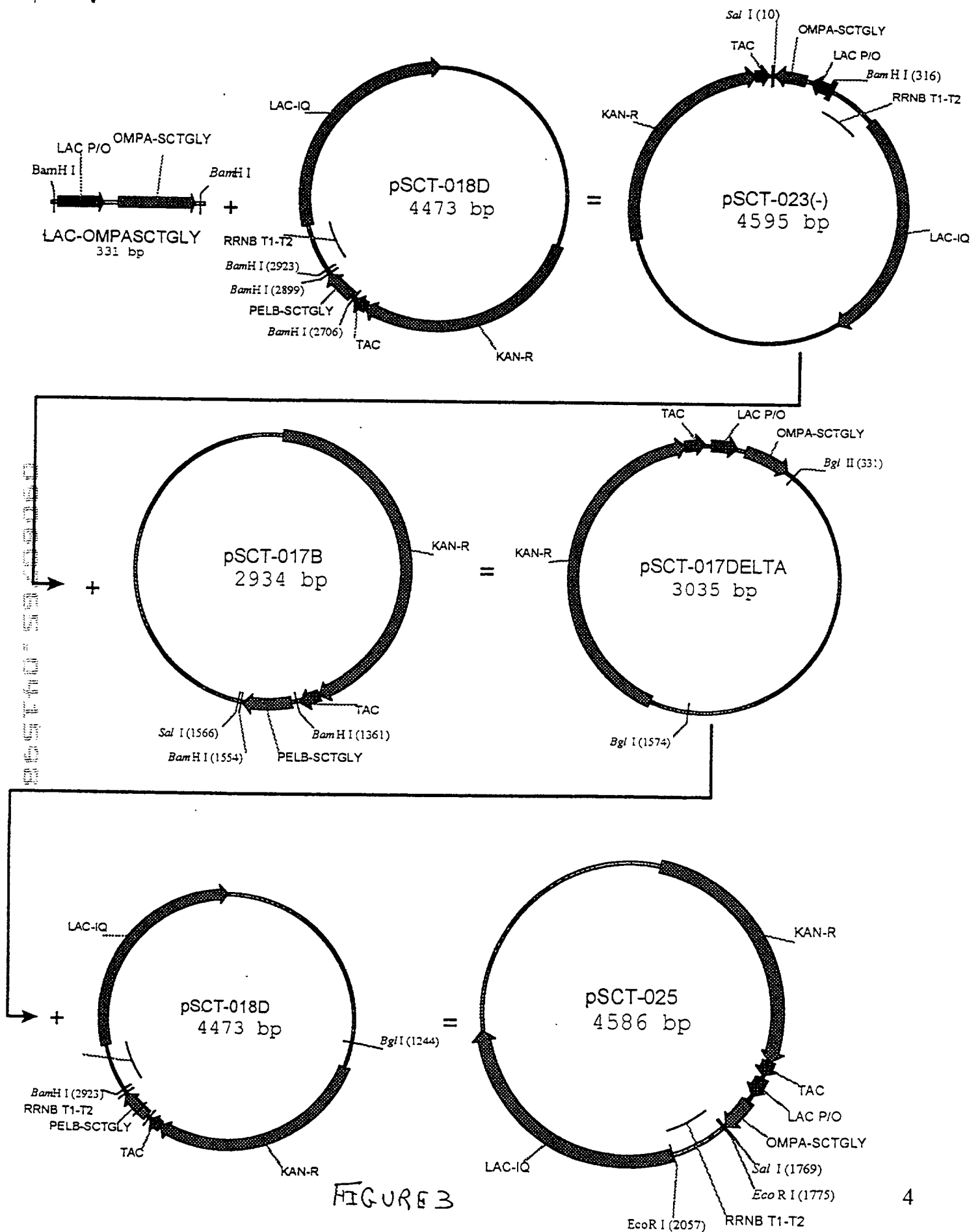


FIGURE 3

Construction of pSCT-029A

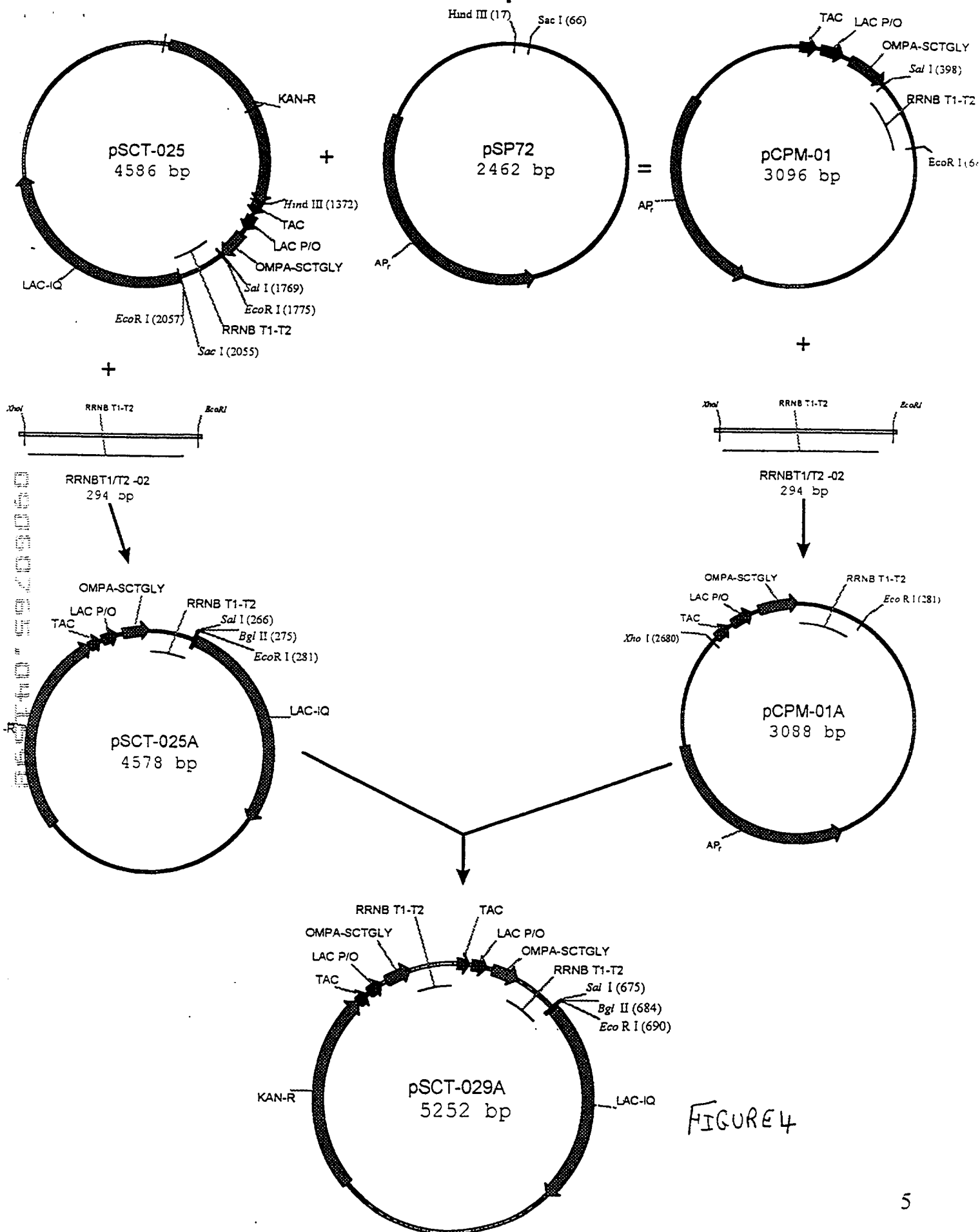
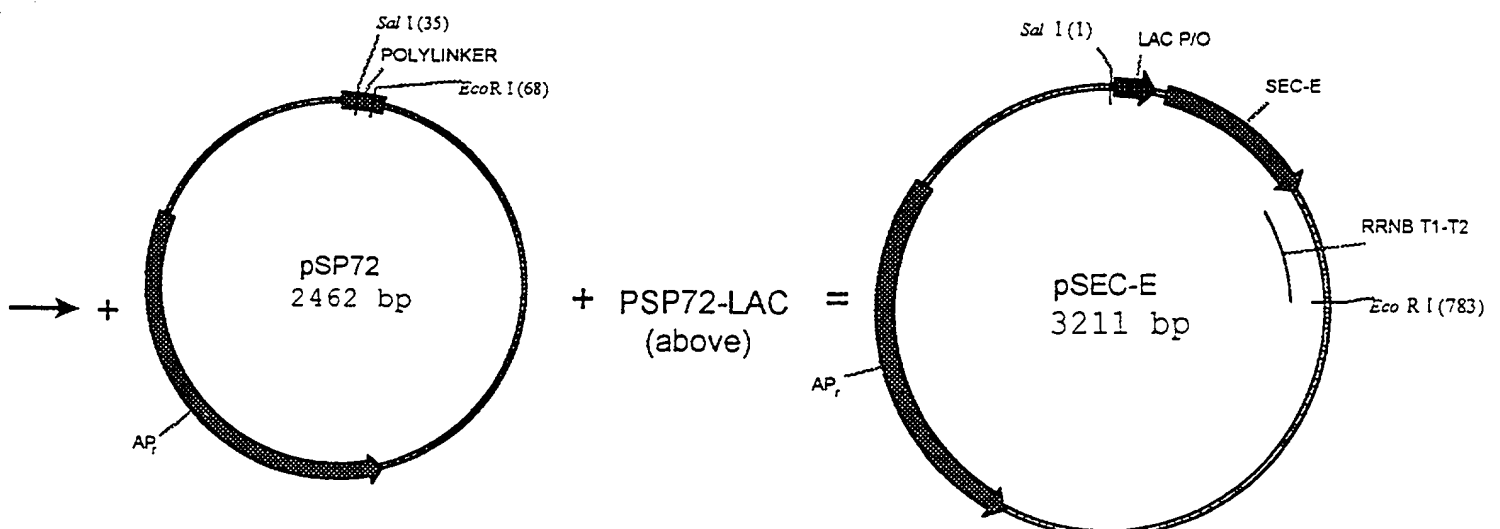
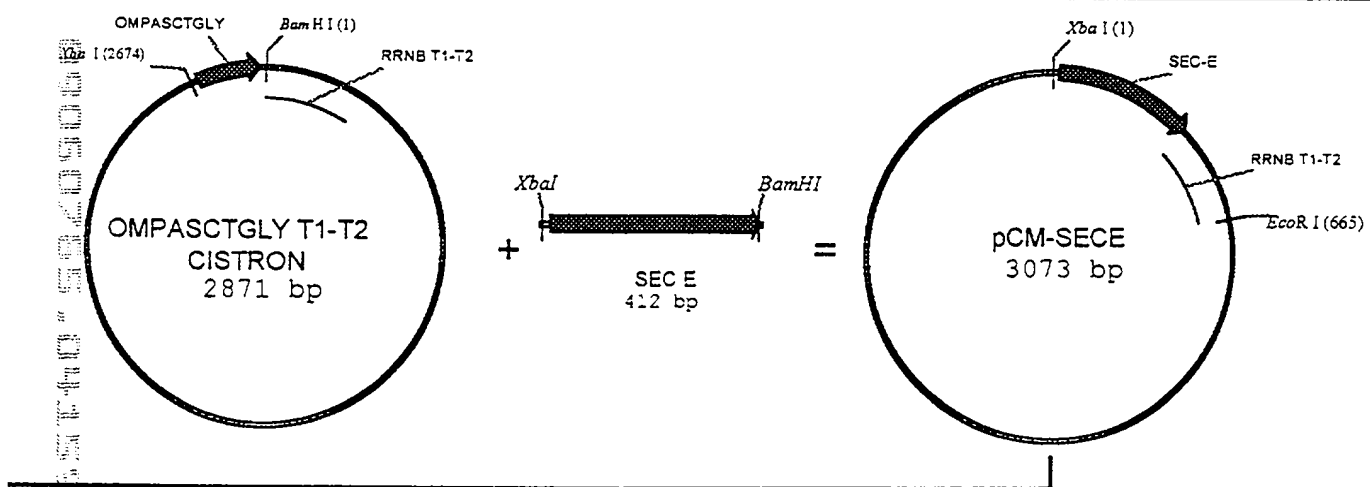
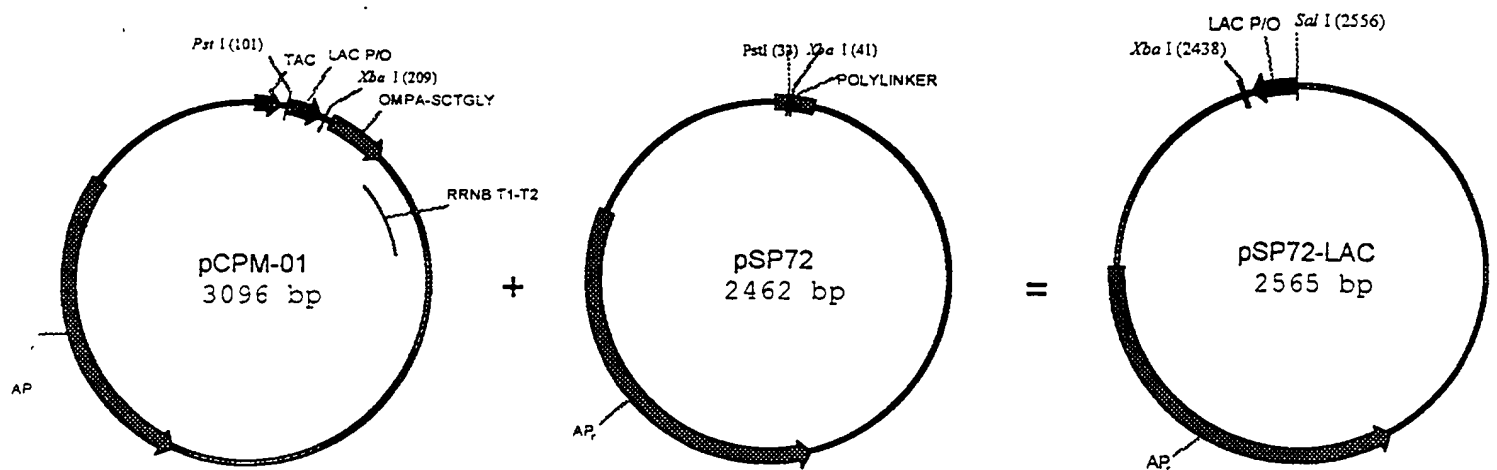


FIGURE 4

Construction of pSEC-E



FIGURES

Construction of pPRLA4

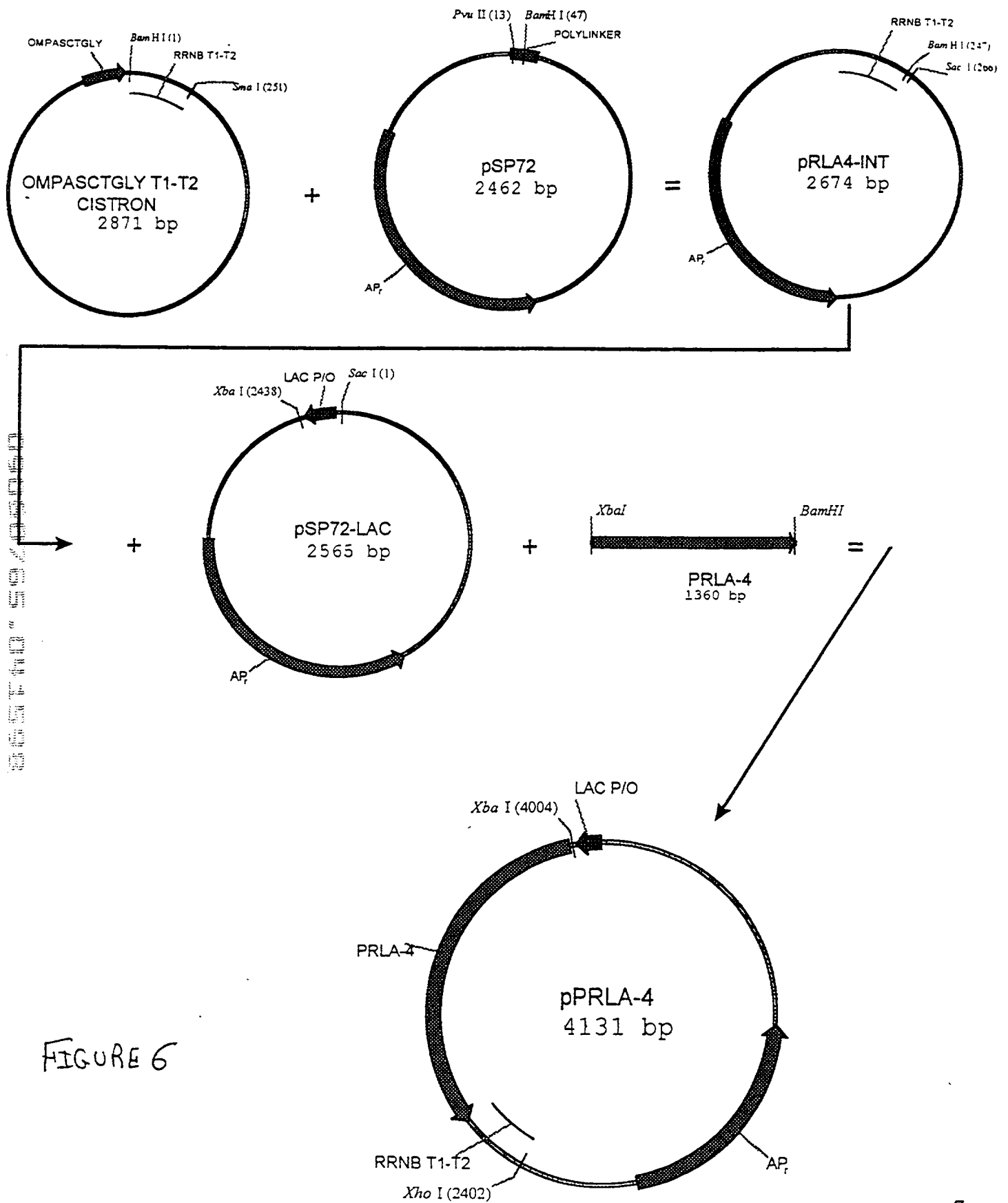


FIGURE 6

Construction of pSEC-EY

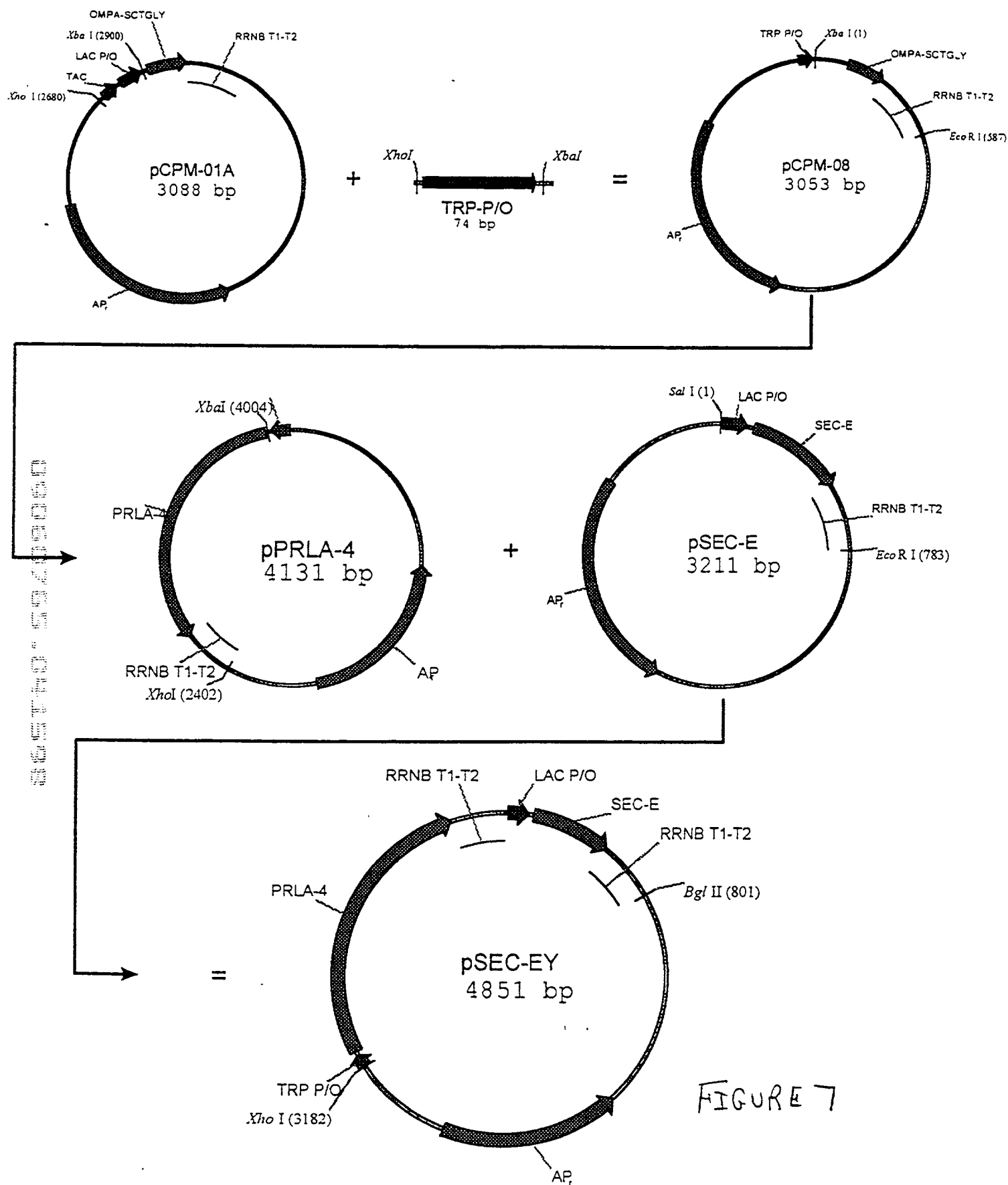


FIGURE 7

Construction of pSCT037 and pSCT038

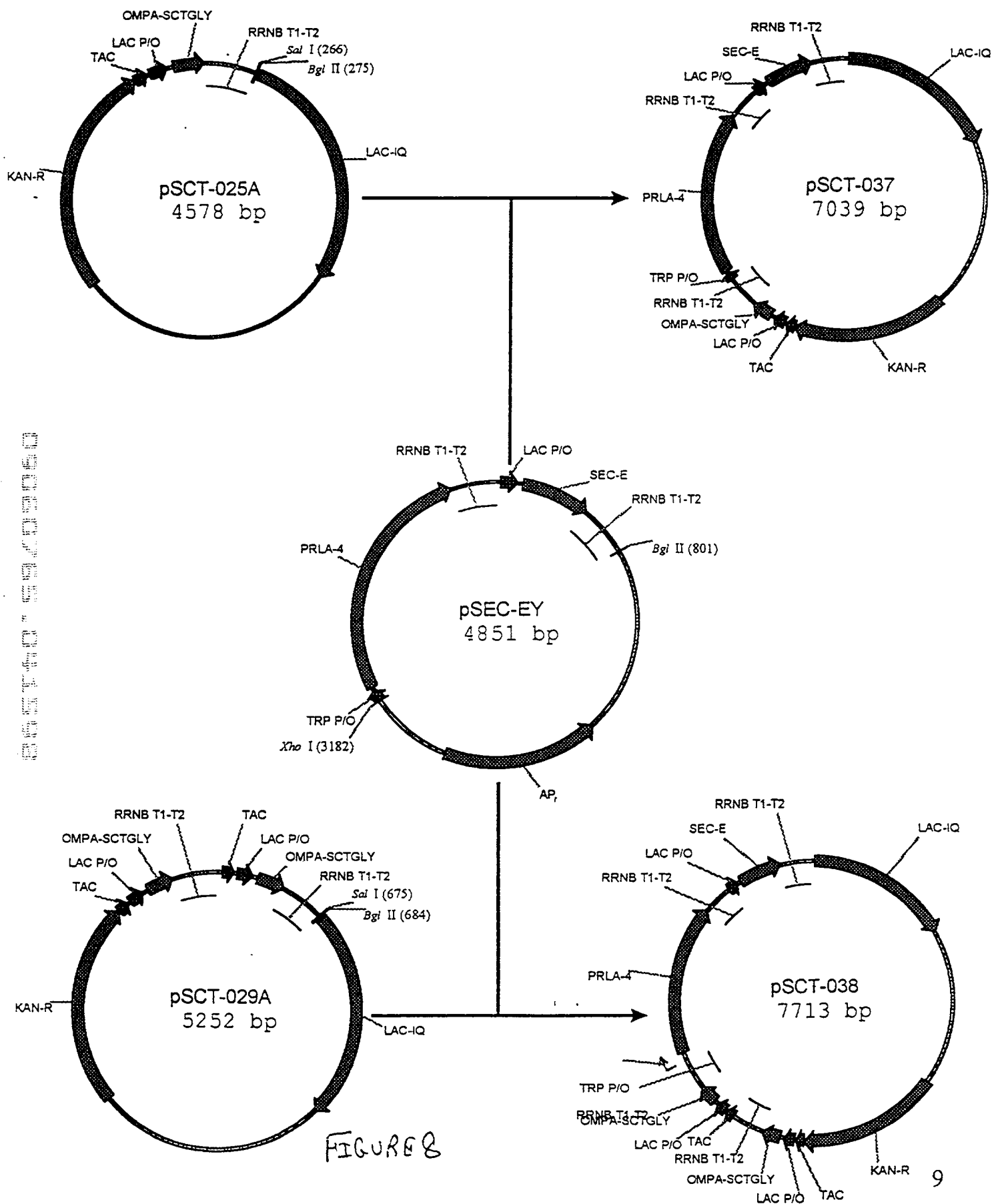


FIGURE 8

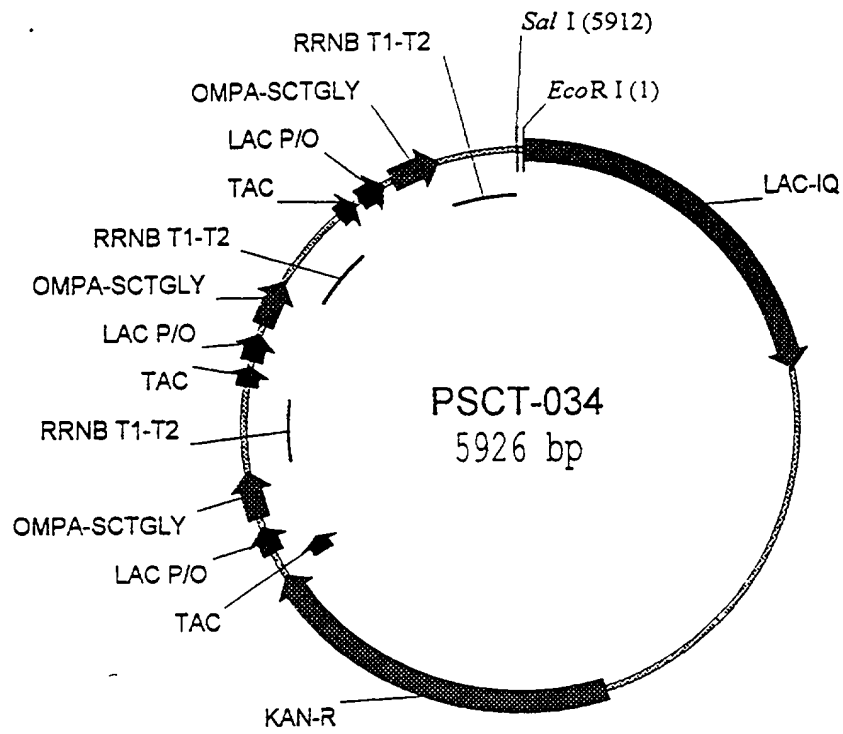


FIGURE 9

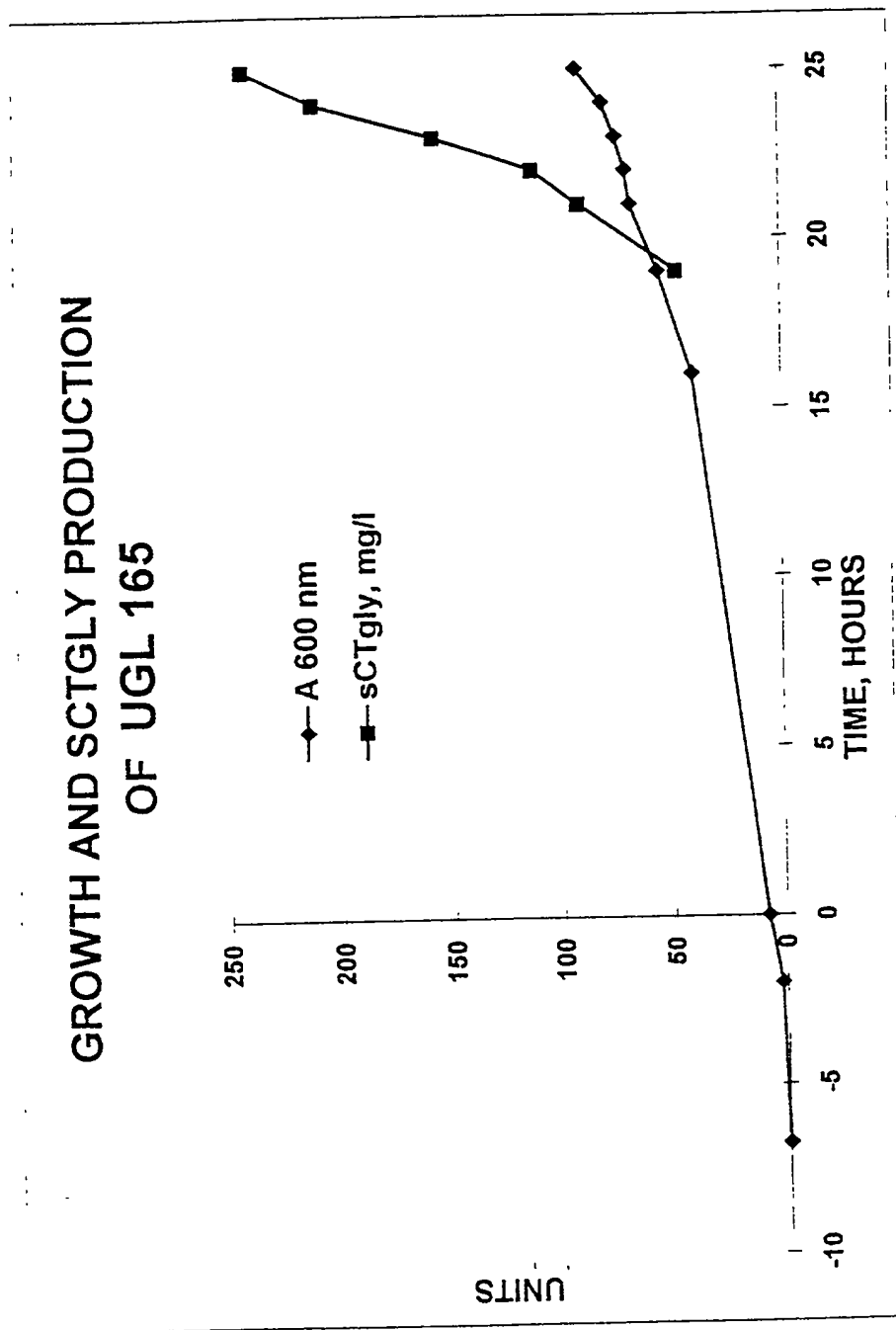


FIGURE 10

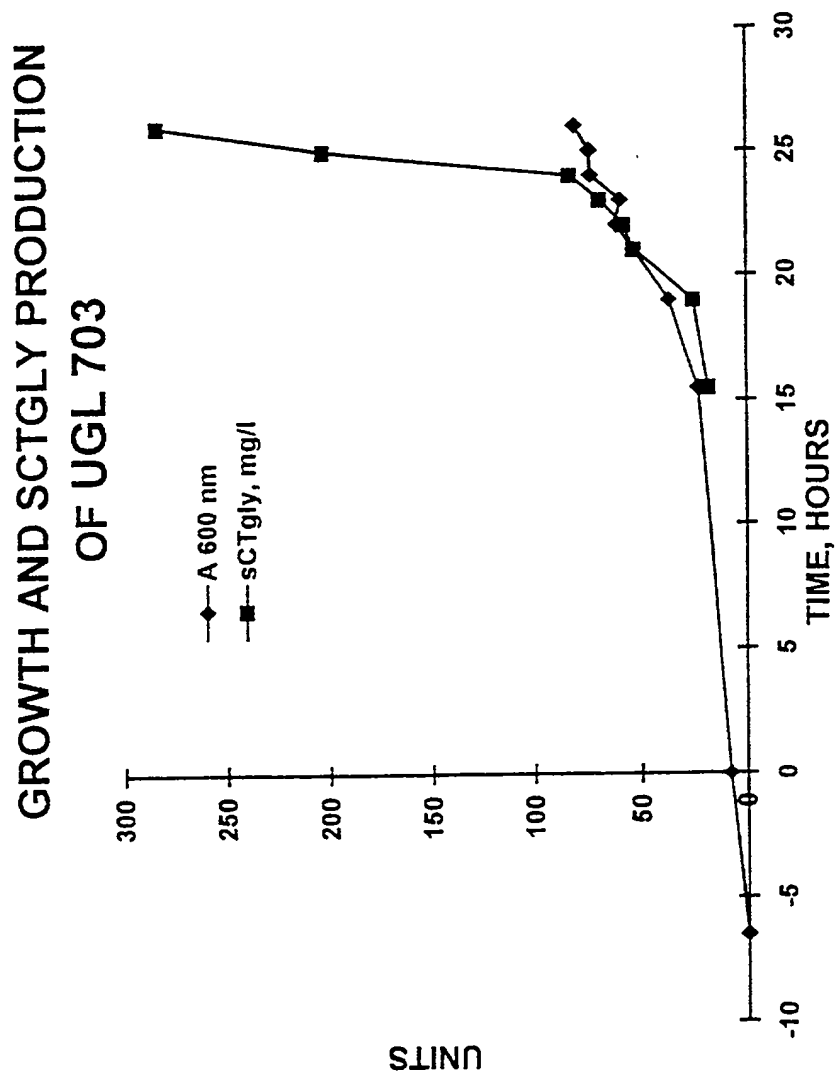


FIGURE 11

FIGURE 14A

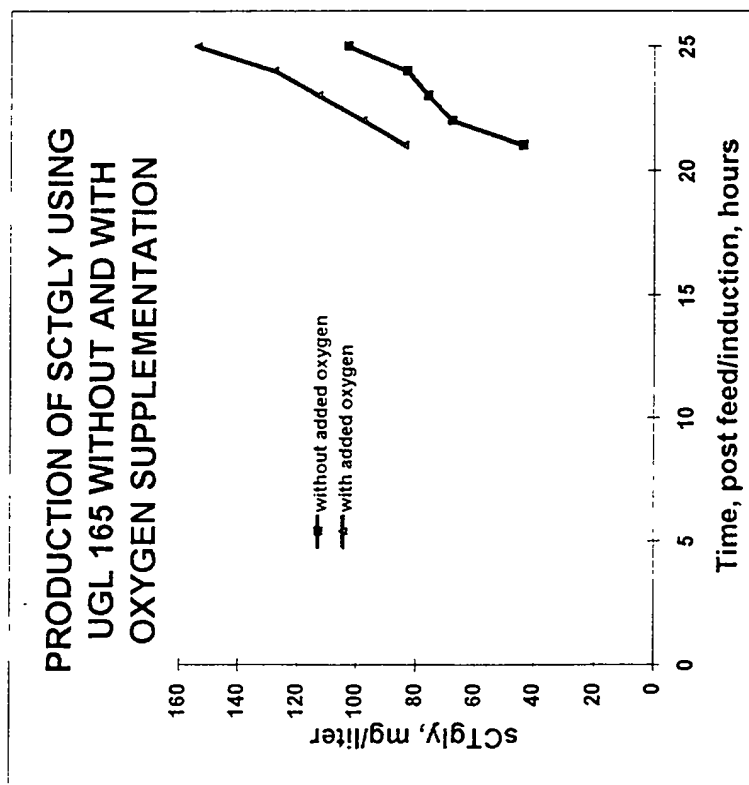


FIGURE 14B

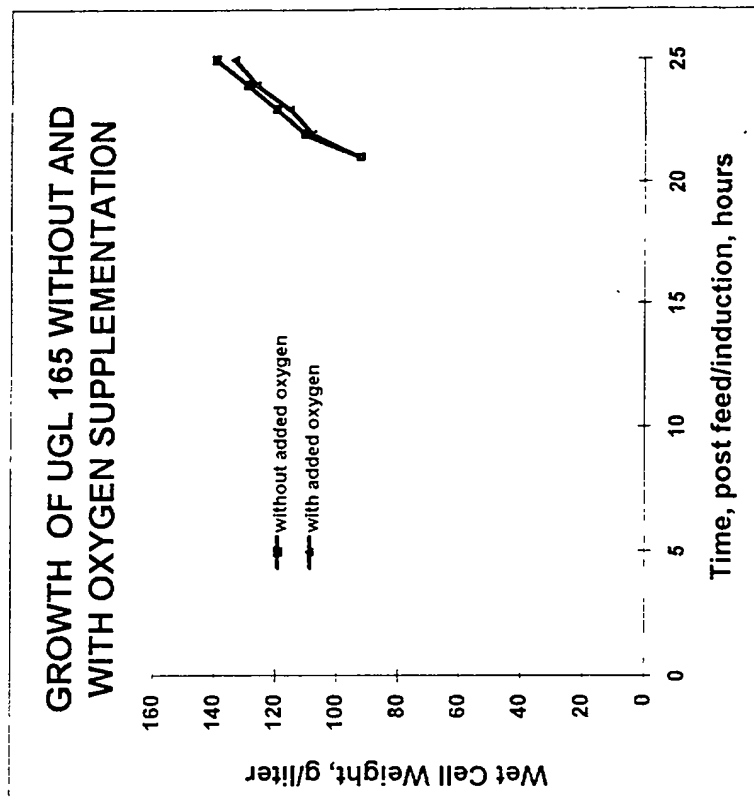


FIGURE 15A

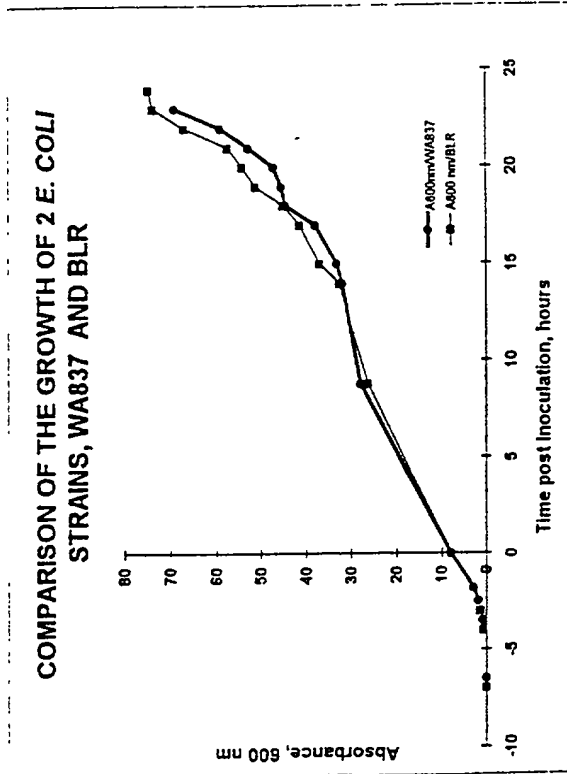
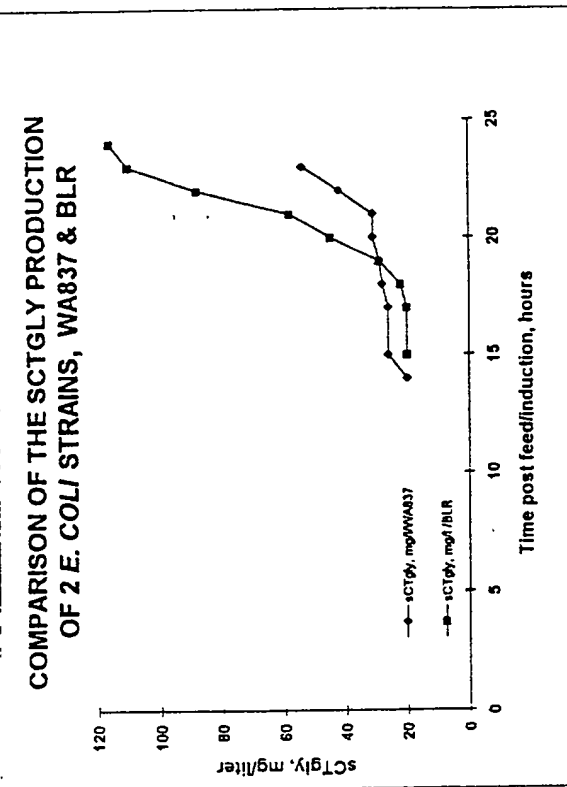


FIGURE 15B



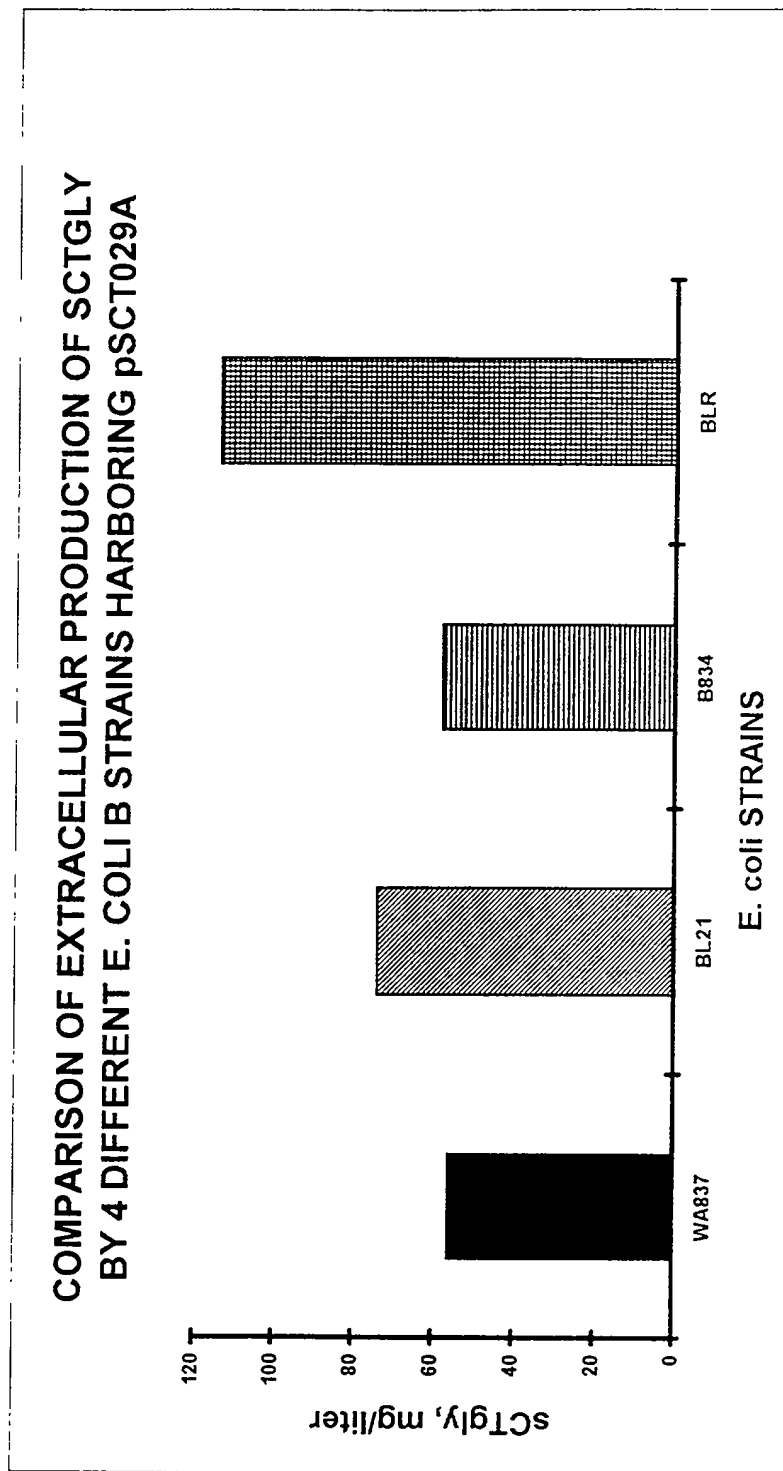
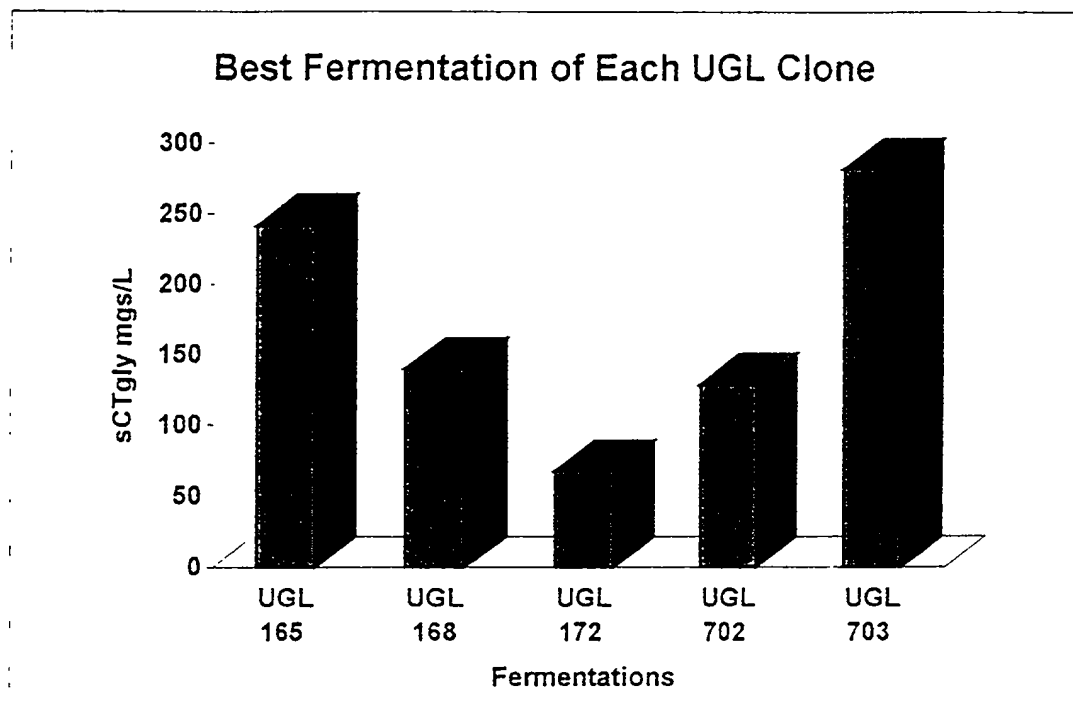


FIGURE 17



UNITED STATES OF AMERICA
COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

OFGS FILE NO.
P/546-192

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

DIRECT EXPRESSION OF PEPTIDES INTO CULTURE MEDIA

the specification of which is attached hereto, unless the following box is checked:

☐ was filed on _____ as United States patent Application Number or PCT International patent application number _____ and was amended on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code §119 of any foreign application(s) for patent or inventor's certificate or United States provisional application(s) listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign or Provisional Application(s)

COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
UNITED STATES	60/043,700	16 APRIL 1997	YES <u>XX</u> NO _____
			YES _____ NO _____

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

UNITED STATES APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby appoint customer no. 2352 OSTROLENK, FABER, GERB & SOFFEN, LLP, and the members of the firm, Samuel H. Weiner - Reg. No. 18,510; Jerome M. Berliner - Reg. No. 18,653; Robert C. Faber - Reg. No. 24,322; Edward A. Meilman - Reg. No. 24,735; Stanley H. Lieberstein - Reg. No. 22,400; Steven I. Weisburd - Reg. No. 27,409; Max Moskowitz - Reg. No. 30,576; Stephen A. Soffen - Reg. No. 31,063; James A. Finner - Reg. No. 30,173; William O. Gray, III - Reg. No. 30,944; Louis C. Dujmich - Reg. No. 30,625 and Douglas A. Miro - Reg. No. 31,643, as attorneys with full power of substitution and revocation to prosecute this application, to transact all business in the Patent & Trademark Office connected therewith and to receive all correspondence.

SEND CORRESPONDENCE TO: **OSTROLENK, FABER, GERB & SOFFEN, LLP** DIRECT TELEPHONE CALLS TO:
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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☒ CONTINUED ON PAGE 2

